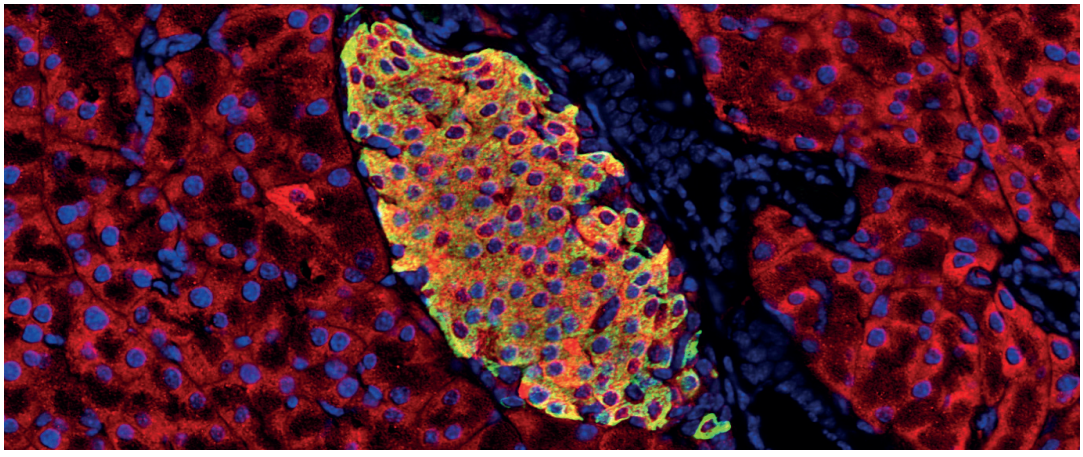
DISSERTATIONES SCHOLAE DOCTORALIS AD SANITATEM INVESTIGANDAM
UNIVERSITATIS HELSINKIENSIS

TATIANA DANILOVA

MANF AS A NEW REGULATOR OF THE UNFOLDED PROTEIN RESPONSE AND MAINTENANCE OF PANCREATIC β-CELLS IN MICE



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DIVISION OF GENETICS
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ACADEMIC DISSERTATION

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Helsinki 2020

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Cover: Immunofluorescence of mouse pancreas: MANF (red), insulin (green) and nuclei (blue).

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Как хорошо! вот сладкий плод ученья!
Как с облаков ты можешь обозреть
Всё царство вдруг: границы, грады, реки.
Учись, мой сын: наука сокращает
Нам опыты быстротекущей жизни —
Когда-нибудь, и скоро, может быть,
Все области, которые ты ныне
Изобразил так хитро на бумаге,
Все под руку достанутся твою —
Учись, мой сын, и легче и яснее
Державный труд ты будешь постигать.

(“Борис Годунов”, 1831, А.С.Пушкин)

Very good! Here's the sweet fruit
Of learning. One can view as from the clouds
Our whole dominion at a glance; its frontiers,
Its towns, its rivers. Learn, my son; 'tis science
Which gives to us an abstract of the events
Of our swift-flowing life. Some day, perchance
Soon, all the lands which thou so cunningly
Today hast drawn on paper, all will come
Under thy hand. Learn, therefore; and more smoothly,
More clearly wilt thou take, my son, upon thee
The cares of state.

(“Boris Godunov”, 1831, A.S. Pushkin)

To my family

ABSTRACT

Neurotrophic factors are small secretory proteins with essential roles in neuronal and non-neuronal tissues. Mesencephalic astrocyte-derived neurotrophic factor (MANF) and cerebral dopamine neurotrophic factor (CDNF) form a distinct family of unconventional neurotrophic factors. MANF and CDNF are endoplasmic reticulum (ER) located, but also secreted proteins. Initially, MANF was discovered as a trophic factor for dopamine neurons *in vitro.* Further studies revealed its protective and restorative properties in different animal disease models such as Parkinson´s disease, spinocerebellar ataxia, brain- and heart-ischemia. MANF is also identified as a protein upregulated in unfolded protein response (UPR) and protecting against ER stress-induced cell death. CDNF was identified based on its homology to MANF and characterized for its ability to protect and restore dopamine neurons in rodent models of Parkinson´s disease. However, the physiological roles of MANF and CDNF in mammals have remained unclear. The main objective of this thesis was, therefore, to study the biological roles of MANF *in vivo* by characterizing the phenotypes of MANF conventional and conditional knockout mice as well as analyzing MANF and CDNF expression in mouse tissues.

Comprehensive expression analysis of MANF mRNA and protein revealed that MANF is widely expressed in most mouse tissues. It is highly expressed in neurons regulating energy homeostasis within the hypothalamus and neurons of other appetite-regulating areas including the brainstem structures and mesolimbic/mesocortical dopamine system. Exceptionally high levels of MANF was observed on peripheral mouse tissues with metabolic function, especially in cells with secretory functions within the endocrine and exocrine glands, suggesting essential roles for MANF in cells with high protein synthesis and secretion. Highest levels of CDNF protein was observed in tissues with high energy production and oxidative function including skeletal muscle, heart, testis and brown adipose tissue.

Silencing of a gene by genetic modifications can give insights on its biological functions *in vivo* in animals. Conventional knockout, as well as conditional knockout mouse models, are valuable tools to discover the roles of a gene in embryonic development and normal physiological homeostasis. In order to study the roles of MANF in mammals, we deloped MANF conventional knockout mice (*Manf*$^{-/-}$), that showed severe growth retardation, poor survival and a progressive postnatal reduction of beta-cell mass resulting in severe insulin-deficient hyperglycemia and diabetes mellitus caused by decreased beta-cell proliferation and increased beta-cell apoptosis. In our further studies, we verified that diabetic phenotype of the *Manf*$^{-/-}$ mice was caused by a lack of MANF in the insulin-producing beta-cells in the pancreas and not in other organs by generating pancreas- and beta-cell-specific conditional *Manf*$^{-/-}$ mice. Our results show that embryonic ablation of MANF in the pancreases of mice and ablation of MANF from beta-cells of adult mice resulted in diabetes. We found that pancreatic islets of conventional and conditional MANF deficient mice displayed chronic activation of the UPR, preceding downregulation of beta-cell markers, indicating unresolved ER stress as one possible cause of beta-cell failure in these mice. Thus, this work shows that MANF is an essential regulator of beta-cell maintenance and UPR in mice.

Diabetes mellitus (DM) is characterized by hyperglycemia and deficient insulin production that is associated with pancreatic beta-cell deficiency and/or dysfunction and leads to massive morbidity and mortality. According to the International Diabetes Foundation (2017), the incidence of diabetes is rising worldwide, and DM will affect 629 million people worldwide by 2045. Most common types of DM are type 1 diabetes (T1D) and type 2 diabetes (T2D). In the case of T1D, the reduction of insulin secretion is caused by the progressive autoimmune destruction of pancreatic beta-cells. T2D is associated with insulin resistance in the peripheral target tissues, followed by the beta-cell dysfunction and loss of pancreatic beta-cell mass. Current technologies for DM treatment do not prevent disease progression, which typically results in devastating long-term diabetic complications. Therefore, new therapies directed for protection pancreatic beta-cell mass and function as well as rejuvenation of the remaining beta-cells are under intensive investigation.

We discovered that MANF protein increased beta-cell proliferation *in vitro* in islets isolated from young and even old mice with very slow beta-cell turnover. MANF protein also rescued mouse beta-cells from thapsigargin-induced apoptosis and ER stress-induced glucotoxicity in culture. Importantly, we found that MANF overexpression in the mouse pancreases mediated by adeno-associated virus vector was able to regenerate beta-cells *in vivo* in a mild low-dose streptozotocin mouse model of diabetes. Hence, these results indicate that MANF is a vital mitogen and protective protein for mouse beta-cells and can thus serve as a potential new regenerative drug for the diabetes therapy.

Furthermore, we identified the decreased number of growth hormone (GH) and prolactin (PRL) expressing cells in the anterior pituitary gland of *Manf*$^{-/-}$ knockout mice associated with increased expression of UPR markers and decreased expression of *Gh* and *Prl* genes. Thus, reduced GH production could be one of the reasons for the growth retardation in *Manf*$^{-/-}$ mice. These results identify indispensable roles for MANF also in endocrine cells of the anterior pituitary besides the pancreatic beta-cells in the *Manf*$^{-/-}$ mice.

Taken together, the results in this thesis provide new critical biological functions of MANF in mouse *in vivo* which can be used to exploit the roles of MANF in human beta-cells and diabetes as well as in endocrine somatotropic cells and growth failure in human.

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Tatiana Danilova
Helsinki, February 2020

TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on two original articles (I, II, III) and unpublished results (manuscript MS IV).

I. **Danilova T**, Galli E, Pakarinen E, Palm E, Lindholm P, Saarma M, Lindahl M. *Mesencephalic Astrocyte-Derived Neurotrophic Factor (MANF) Is Highly Expressed in Mouse Tissues With Metabolic Function.* Front Endocrinol (Lausanne) 2019; 10, 765.

II. Lindahl M, **Danilova T**, Palm E, Lindholm P, Voikar V, Hakonen E, Ustinov J, Andressoo JO, Harvey BK, Otonkoski T, Rossi J, Saarma M. *MANF is indispensable for the proliferation and survival of pancreatic beta cells*. Cell Rep 2014;7(2):366-375

III. **Danilova T**, Belevich I, Li H, Palm E, Jokitalo E, Otonkoski T, Lindahl M. *MANF Is Required for the Postnatal Expansion and Maintenance of Pancreatic beta-cell Mass in Mice*. Diabetes 2019;68(1):66-80

IV. **Danilova T**, Blom S, Ropponen T, Pitkänen K, Lindahl M. *Fully automated histological analysis of mouse pancreas using deep learning.* Manuscript

In addition, some unpublished results are presented.

ABBREVIATIONS

6-OHDA - 6-hydroxydopamine
AAV - adeno-associated virus
ACTH - adrenocorticotropic hormone
α-MSH - α-melanocyte-stimulating hormone
ARTN - Artemin
ASK1 - apoptosis signal-regulating kinase
ATF6 - activating transcription factor 6
ATF6f - cytosolic domain fragment of ATF6
BAT - brown adipose tissue
BDNF - brain-derived neurotrophic factor
CDNF - cerebral dopamine neurotrophic factor
CLAMS - comprehensive laboratory animal monitoring system
CNN - convolutional neural network
CNS - central nervous system
DM - diabetes mellitus
dMCAO - cortical stroke model of cerebral artery occlusion
EAE - experimental autoimmune encephalomyelitis
EGF - epidermal growth factor
ER - endoplasmic reticulum
ERAD - ER-associated degradation
ERSE - ER stress-responsive elements
ES - embryonic stem
FFAs - free fatty acids
FGF - fibroblast growth factor
FSH - gonadotropes follicle stimulating hormone
GADA65a - glutamate acid decarboxylase
GADD34 - growth arrest and DNA damage protein
GDM - gestational diabetes mellitus
GFLs - GDNF family ligands
GH - growth hormone
GLP-1 - glucagon-like peptide 1
GLUT2 - glucose transporter 2
GRP78 - glucose-regulated protein, 78kD
GTT - glucose tolerance test
H9c2 - rat myoblast cell line
HFD - high-fat diet
HSP - heat shock proteins

IAPP - islet amyloid polypeptide
IBMX - 3-isobutyl-1-methylxanthine
IGF - insulin-like growth factor
IHC - immunohistochemistry
iNOS - inducible isoform nitric oxide synthase
INS1-1E - rat insulinoma-derived clonal cell line
IP_3 - inositol-1, 4, 5-trisphosphate
IRE1 - inositol-requiring enzyme 1
ISH - *in situ* hybridization
ITT - insulin tolerance test
JNK - c-Jun N-terminal kinase
LADA -latent autoimmune diabetes in adults
LH - luteinizing hormone
Lhβ - luteinizing hormone beta
MANF - mesencephalic astrocyte-derived neurotrophic factor
MED - multiple epiphyseal dysplasias
MIDY - mutant INS-gene diabetes of youth
MLD-STZ - multiple low-dose streptozotocin
NDM - neonatal diabetes
NGF - nerve growth factor
NMR - nuclear magnetic resonance
NO - nitric oxide
NOD - non-obese diabetic mice
NRF2 - nuclear factor erythroid 2-related factor
NRTN - neurturin
NT-3 - neurotrophin-3
NT-4 - neurotrophin-4
NTFs - neurotrophic factors
OGD - oxygen-glucose deprivation
PARP - poly (ADP-ribose) polymerase
PD - Parkinson’s disease
PDGF - platelet-derived growth factor
PDX1 - pancreatic and duodenal homeobox 1
PERK - PKR-like ER kinase
Pit1 - pituitary-specific positive transcription factor 1
PKC - protein kinase C
PNDM - permanent neonatal diabetes
PNS - peripheral nervous system
PP - pancreatic polypeptide cells

PP1 - protein phosphatase 1
PRL - prolactin
PSPN - persephin
PTH - parathyroid hormones
RIDD - IRE1-dependent decay
RIP-LCMV-GP - rat insulin promoter-lymphocytic choriomeningitis virus-glycoprotein
ROS - reactive oxygen species
RT-PCR - reverse transcription polymerase chain reaction
SCA17 - spinocerebellar ataxia 17
SCG - superior cervical ganglion
sp - spliced
T1D - type 1 diabetes
T2D - type 2 diabetes
T3 - triiodothyronine
T4 - thyroxine
TBP - TATA-box binding protein
TGFβ - transforming growth factor β
TH - tyrosine hydroxylase
Tmx - tamoxifen
TNDM - transient neonatal diabetes
TRAF2 - scaffold protein tumor necrosis factor receptor-associated factor 2
TrkC - tropomyosin receptor kinase C
TRIB3 - tribbles homolog 3
TSH - thyroid-stimulating hormone
TUNEL - terminal deoxynucleotidyl transferase dUTP nick end labeling
TXNIP - thioredoxin-interacting protein
U2OS - human bone osteosarcoma epithelial cell line
UPR - unfolded protein response
VEGF - vascular endothelial growth factor
VMCL1 - type-1 astrocyte ventral mesencephalic cell line 1
WAT - white adipose tissue
WRS - Wolcott-Rallison syndrome
WS1 - Wolfram syndrome 1
Xbp1 - X-box binding protein 1

REVIEW OF LITERATURE

1. Neurotrophic factors and growth factors

Neurotrophic factors (NTFs) are small secretory proteins that during development and adulthood promote the survival of neurons, maintain their neuritic contacts and regulate neuronal plasticity. Outside the nervous system, NTFs have essential functions with therapeutic potential for the treatment of various chronic neurological and metabolic disorders.

In the early 1950's the first growth factors discovered by developmental biologist Rita Levi-Montalcini and biochemist Stanley Cohen were the nerve growth factor (NGF) and epidermal growth factor (EGF) respectively. Currently, there are four major NTF families: neurotrophins, the GDNF family ligands (GFLs), the neuropoietic cytokines, and the CDNF/MANF family. In addition to these families, various other growth factors families like transforming growth factor β (TGF β), vascular endothelial growth factor (VEGF), insulin-like growth factors (IGF) and fibroblast growth factor (FGF) have neurotrophic activities (Grothe and Timmer, 2007; Zacchigna et al., 2008).

Neurotrophin family is represented by NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4), signaling of which is critically important for the development and maintenance of the nervous system (Huang and Reichardt, 2001; Lu et al., 2005). Neurotrophin family members were shown to regulate energy homeostasis (Fargali et al., 2012). NGF and BDNF are also known to regulate the immune cell activity (Calabrese et al., 2014; Minnone et al., 2017). NT-3 and its high-affinity tropomyosin receptor kinase C (TrkC) regulate heart development by modulating the replication of embryonic cardiomyocytes (Donovan et al., 1996; Lin et al., 2000). Moreover, BDNF was shown to be involved in regulation of glucose metabolism in mice and humans with type 2 diabetes (Tonra et al., 1999; Krabbe et al., 2007).

The four members of the GFLs include GDNF, Neurturin (NRTN), Artemin (ARTN) and Persephin (PSPN). The GFLs play an essential role in the development, differentiation, and maintenance of various neurons (Airaksinen and Saarma, 2002). In addition, GDNF is important for kidney development and spermatogonial differentiation (Pichel et al., 1996; Meng et al., 2000). Moreover, recent studies identified protective and mitogenic functions of GDNF for pancreatic beta-cells (Mwangi et al., 2008).

Mesencephalic astrocyte-derived neurotrophic factor (MANF) and cerebral dopamine neurotrophic factor (CDNF) are a diverse family of evolutionarily conserved neurotrophic factor family (Petrova et al., 2003; Lindholm et al., 2007; Palgi et al., 2009). MANF was initially purified from the of rat type-1 astrocyte ventral mesencephalic cell line 1 (VMCL1) culture medium and discovered its protective properties on embryonic dopaminergic neurons *in vitro* (Petrova et al., 2003). The second member of the trophic factor family, CDNF, was determined by a bioinformatics and biochemical analysis based on its homology to MANF (Lindholm et al. 2007). A single orthologue of mammalian MANF and CDNF was identified

in invertebrates including fruit fly, pea aphid and marine sponge and revealed about 50% identity with human MANF.

1.1 Sequence and three-dimensional structure of MANF and CDNF

MANF and CDNF are both 18 kDa secreted proteins, highly soluble and monomeric in neutral solution (Lindholm et al., 2007; Mizobuchi et al., 2007; Hoseki et al., 2010; Hellman et al., 2011; Latge et al., 2015). Sequence analysis revealed that human MANF and CDNF are 179 and 187 amino acid residues long, respectively.

Both proteins contain a predicted signal sequence and biologically active mature region. The pre-region of MANF is 21 amino acids long, and the mature form of MANF contains 158 amino acid residues (Petrova et al., 2003). CDNF, in turn, encodes 26 amino acids long signal sequence and 161 residues long mature region (Lindholm et al., 2007). Full-length human CDNF and MANF sequences are 59% amino-acid identical. Differently from other classical NTFs, CDNF and MANF lack the pro sequence, indicating that enzymatic cleavage is not required for their activation. Moreover, both MANF and CDNF have eight conserved cysteine residues that form four disulfide bridges, while neurotrophins and other GFLs have seven (Airaksinen and Saarma, 2002; Petrova et al., 2003; Lindholm et al., 2007).

Several groups have studied the three-dimensional structure of MANF and CDNF using X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy in aqueous solution (Parkash et al., 2009; Hoseki et al., 2010; Hellman et al., 2011; Latge et al., 2015). Based on these studies, MANF and CDNF have a similar 3D structure. They contain two distinct domains connected with a short linear sequence. NMR studies of the full-length proteins confirmed the folding of MANF and CDNF into two domains linked with a flexible loop (Hellman et al., 2011; Latge et al., 2015). The 12 kDa N-terminal domain is structurally similar to saposin-like proteins (Parkash et al., 2009), a family of proteins with ability to interact with lipids (Bruhn, 2005). A recent study demonstrated that MANF but not CDNF bind to lipid sulfatides in the membrane of cell membranes of *C. elegans* and mammalian cells (Bai et al., 2018). Moreover, the ability of MANF to bind sulfatides was reduced by mutation at its N-terminal part, indicating that MANF directly binds sulfatides via its N-terminal part.

The 6 kDa C-terminal domain of MANF and CDNF resembles the SAP-like domain in Ku70, which is an inhibitor of the pro-apoptotic Bax protein (Hellman et al., 2011; Latge et al., 2015). The C-terminal MANF protected neurons against Bax-induced apoptosis *in vitro* efficiently as Ku70 (Hellman et al., 2011), although the mechanism was not studied. The SAP-like domain of MANF and CDNF might bind to DNA (Hellman et al., 2011).

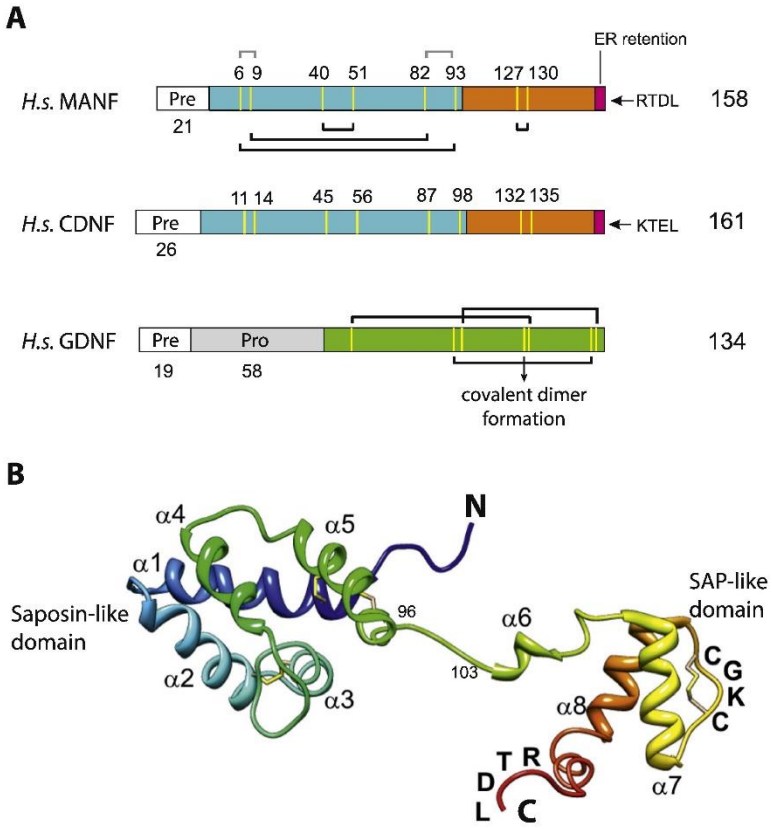

Figure 1. (A) The schematic image represents a comparison of the human MANF and CDNF structures with that of GDNF. The N-terminal saposin-like and C-terminal SAP-like domains of MANF and CDNF are shown in blue and orange, respectively. Yellow bars are representing conserved cysteine residues according to the mature protein sequence. The formation of cysteine bridges is shown in black connecting lines and proposed disulfide bridges. Red bars are indicative of ER retention signal. Differently from MANF and CDNF, the primary structure of GDNF contains a pro sequence, indicating that enzymatic activation of pro-region is needed for its activation. Mature GDNF is represented in green and disulfide bonds modeling three disulfide bridges and a cysteine motif are marked with black lines. Two GDNF molecules form a covalently linked homodimer. Disulfide bridge and a cysteine residue implicated in this process are shown by an arrow. H.s.; Homo sapiens. **(B)** Crystal structure of human MANF containing of the N-terminal saposin-like domain and the C-terminal SAP-like domain. Alpha-helices (a) are marked from the beginning of the N-terminus. The area between residues 96–103 represents the linker region. CGKC loop motif, ER retention signal RTDL, N - amino-terminus, C, carboxy-terminus are marked on the scheme. The image is adapted from (Lindahl et al., 2017)

Another study identified the interaction of C-terminal domain of MANF with the DNA-binding subunit of NF-κB p65, although the exact binding mechanism was not addressed (Chen et al., 2015a).

Conserved motifs of MANF and CDNF include four disulfide bridges and an ER retention signal at their C-terminal domains. Two cysteines in the conserved CXXC motif that can be found in the catalytic centers of redox enzymes, form one disulfide bridge. Although numerous attempts have failed to demonstrate the oxidoreductase activity of MANF (Mizobuchi et al., 2007; Hartley et al., 2013; Matlik et al., 2015), a mutational analysis of the CXXC motif did reveal that this motif is indispensable for the cytoprotective function of MANF (Lindstrom et al., 2013; Matlik et al., 2015).

Moreover, the last four amino acids of the C-terminal domain of MANF and CDNF, RTDL and KTEL respectively, resemble the KDEL ER retention signal that can mediate their binding to KDEL receptors and facilitate their ER retention (Raykhel et al., 2007). Both MANF and CDNF are intracellular proteins that predominantly reside within the ER (Apostolou et al., 2008; Tadimalla et al., 2008; Sun et al., 2011; Henderson et al., 2013; Fernandez et al., 2014; Matlik et al., 2015). Deletion of the RTDL motif from MANF sequence results in enhanced secretion of MANF and its mislocalization from the ER to Golgi both in cultured cells and neurons (Glembotski et al., 2012; Henderson et al., 2013; Henderson et al., 2014). Similarly, ablation of KTEL motif from CDNF resulted in the increased secretion of overexpressed CDNF (Norisada et al., 2016), indicating diminished ER retention.

1.2 MANF and CDNF expression in the mouse and human tissues

Analysis of MANF expression was addressed by different techniques, such as *in situ* hybridization (ISH), reverse transcription polymerase chain reaction (RT-PCR), Western blotting and immunohistochemistry (IHC) analyses (Lindholm et al., 2008; Wang et al., 2014; Yang et al., 2014a; Tseng et al., 2017; Yang et al., 2017). During embryonic development, wide MANF expression was observed in the mouse CNS and peripheral tissues (Lindholm et al., 2008). High expression of MANF was detected in the dorsal root ganglia, trigeminal ganglia, and superior cervical ganglion by IHC. In non-neuronal tissues, high levels of MANF was identified in the pancreas, salivary gland, liver and the cartilage cells during embryo development.

Broad expression of MANF protein and mRNA have been observed in mouse tissues postnatally. Within the rodent brain, MANF expression was mainly detected in the neuronal cells and not astrocytes by IHC (Lindholm et al., 2008; Tseng et al., 2017). ISH revealed *Manf* mRNA expression in different postnatal and adult rodent brain regions including olfactory bulb, cortex, hippocampus, hypothalamus, substantia nigra and spinal cord. MANF positive neurons were co-expressed with tyrosine hydroxylase (TH)-positive neurons in substantial nigra in both mouse and rat (Lindholm et al., 2008; Wang et al., 2014). Interestingly, MANF expression in the rat brain was the highest at two-weeks of age with decreasing levels thereafter (Wang et al., 2014). Moreover, MANF co-expressed with calbindin-positive Purkinje cells (Yang et al., 2014a).

Outside the CNS, it was shown that MANF protein is broadly expressed in the non-neuronal cells of adult mouse tissues. Especially MANF was strong in the secretory tissues such as pancreatic exocrine cells and endocrine islets of Langerhans, salivary gland and testis (Mizobuchi et al., 2007; Lindholm et al., 2008), indicating unique properties of MANF in the cells with high protein synthesis and secretion.

Studies of MANF expression in the human tissues have not been extensive (Lindholm et al., 2008). MANF mRNA was detected in several human brain regions and human peripheral tissues similarly to mouse as previously reported by Lindholm et al (Lindholm et al., 2008). Recent findings revealed the presence of MANF in the human blood serum (Galli et al., 2016).

Previously Lindholm et al. demonstrated that *Cdnf* mRNA and protein levels in the mouse tissues had been shown to be generally lower than MANF levels (Lindholm et al., 2007). *Cdnf* mRNA expression was detected in the mouse CNS as well as adult human brain studied. CDNF expression was observed in the mouse neurons of cortex, striatum, hippocampus, substantia nigra, locus coeruleus and Purkinje cells of the cerebellum. In contrast to MANF, no CDNF expression was observed in dopaminergic neurons of the substantia nigra. Additionally, authors detected CDNF mRNA in all analyzed non-neuronal tissues in human and mouse, where highest levels of CDNF were found in heart, muscle and testis (Lindholm et al., 2007).

To conclude, both MANF and CDNF are ubiquitously expressed in mouse and human neuronal and non-neuronal tissues. However, the pattern and the levels of their expression differs from each other, indicating distinct functions of these homologous proteins.

1.3 Functions of MANF

1.3.1 MANF therapeutic potential in rodent models of Parkinson's disease

Parkinson's disease (PD) is a neurodegenerative disease associated with specific loss of dopamine neurons in substantia nigra pars compacta and their projections to the caudate putamen (in rodents striatum) (Andressoo and Saarma, 2008). The motor symptoms of PD first appear when 40 to 50% of the dopamine neurons are lost and include rigidity, tremor, involuntary, and slowed movement (Lang and Lozano, 1998; Dauer and Przedborski, 2003). It remains unknown what is the primary cause of the dopamine neuron degeneration in PD. According to the current view PD is triggered by a combination of underlying genetic predisposition and environmental exposures.

The 6-hydroxydopamine (6-OHDA) model of PD was the first established rodent model of PD, and has been extensively used to investigate Parkinsonism ever since 1968 (Simola et al., 2007). MANF was shown to protect and promote functional recovery of dopamine neurons in rodent PD models. Intrastriatal delivery of human recombinant MANF effectively protected dopamine neurons and improved locomotor behavior in 6-OHDA-induced rat model of PD (Voutilainen et al., 2009), although these results were not reproduced in a more severe 6-OHDA model when MANF was chronically infused (Voutilainen et al., 2011). Intrastriatal lentiviral delivery of MANF had no effect in a rat model of PD induced by the 6-OHDA (Cordero-Llana et al., 2015), but the authors did not document MANF expression in the midbrain. Although intranigral delivery rescued dopamine neurons in substantia nigra pars compacta, it did not affect the TH-fiber density in striatum neither improved the behavioral deficit. However, MANF expression was not demonstrated. Other studies identified that AAV9-mediated delivery of MANF to the striatum of rat brains protected the neurons from 6-OHDA (Hao et al., 2017). AAV9-MANF promoted recovery of the TH-positive dopamine neurons in the substantia nigra, leading to increased TH-fiber density in the striatum and improved locomotor behavior.

The mechanisms of protective action of MANF is unknown but current data points towards several possible mechanisms of action. Studies revealed that MANF rescued SH-

SY5Y cells from the 6-OHDA induced apoptosis by inhibiting autophagy through the initiation of the AMPK/mTOR cascade (Zhang et al., 2017b). MANF-mediated protection against 6-OHDA-induced cytotoxicity was suggested to be regulated via the PI3K/Akt/GSK3β pathway by regulating the expression of nuclear factor erythroid 2-related factor (Nrf2) (Zhang et al., 2017c). Moreover, MANF protected SH-SY5Y cells from the 6-OHDA induced apoptosis and overexpressed α-synuclein via upregulation of some ER stress markers (Huang et al., 2016; Sun et al., 2017). In addition, the protective roles of viral vector delivered MANF on 6-OHDA treated cells corresponded with decreased unfolded protein response (UPR) (Hao et al., 2017). Taken together, although some mechanistic insights were proposed, the exact mode of MANF therapeutic action remains unclear.

1.3.2 MANF therapeutic potential in a mouse model of spinocerebellar ataxia

Spinocerebellar ataxia 17 (SCA17) is a neurodegenerative disease characterized by selective degeneration of the Purkinje cells in the cerebellum (Toyoshima et al., 1993). SCA17 is caused by a poly-glutamine expansion of the transcription factor of TATA-box binding protein (TBP). Decreased expression of MANF in the cerebellar Purkinje cells followed by their degeneration was observed in a conditional knock-in mouse of mutated TBP (Yang et al., 2014a). Degeneration of Purkinje cells was diminished by MANF overexpression via protein kinase C (PKC)-dependent signaling (Yang et al., 2014a), suggesting a protective role for MANF in Purkinje cells. To date, no other studies revealing MANF functions via PKC pathway are known. Moreover, MANF concentrations (1.5 mg/ml) used in this study are not physiological. Recent studies revealed that ER stress contributes to the pathogenesis of SCA17 and MANF suppresses ER stress consequently diminishing the mutant TBP toxicity (Guo et al., 2018), indicating that MANF exerts is beneficial effects through dampening ER stress.

1.3.3 MANF roles in inflammation

Inflammation is a major pathological event in many chronic and degenerative diseases (Chen et al., 2018). It is the response of the immune system to harmful stimuli, which helps to alleviate infections, initiates clearance of the damaged cells and pathogens, and repair of injured tissue (Medzhitov, 2010). These processes lead to the recovery of tissue homeostasis and the termination of the acute inflammation. However, if not resolved, acute inflammation becomes chronic (Nathan and Ding, 2010), contributing to a variety of chronic inflammatory diseases including auto-immune diabetes, cardiovascular diseases, atherosclerosis, rheumatoid arthritis, and cancer (Sugimoto et al., 2016). Inflammatory responses also occur in neurodegenerative diseases like PD, Alzheimer's diseases, stroke, and epilepsy (Chen et al., 2018). Chronic inflammation associated with aging disrupts metabolic function in mammals, leading to obesity and diabetes (Barzilai et al., 2012). Hence, there is a great need for developing new potential anti-inflammatory drugs.

MANF has an essential role in the modulation of immune responses. It was documented that MANF expression is detected in immune cell of invertebrates and vertebrates, and its expression is upregulated upon inflammatory responses (Chen et al., 2015a; Liu et al., 2015a;

Neves et al., 2016; Sereno et al., 2017). MANF rescued astrocytes from oxygen-glucose deprivation (OGD) and promotes cell survival by reducing the expression and secretion of proinflammatory cytokines IL-1β, IL-6, and TNF-α induced by ER stress and detected after OGD (Zhao et al., 2013). Moreover, treatment with MANF led to decreased levels of GRP78 and NF-κB p65 that are also induced by OGD. Other studies identified that MANF suppresses the transcriptional activities of NF-κB inflammatory pathways in synoviocytes (Chen et al., 2015a). Moreover, MANF has been shown to promote anti-inflammatory activation of immune cells in both flies and mice. MANF expression promoted by PDGF-A in injured retina cells in flies and mice induced alternative activation of innate anti-inflammatory M2-type immune cells, thereby promoting retinal tissue repair and enhancing the success of photoreceptor replacement therapies (Neves et al., 2016). Thus MANF seems to act as an anti-inflammatory agent by reducing ER stress and production of pro-inflammatory cytokines and also by affecting alternative activation of anti-inflammatory immune cells.

MANF expression was shown to decline with aging in flies, mouse tissues (liver, muscle, fat, and skin) and human skin (Sousa-Victor et al., 2019). Levels of MANF were reduced in both mouse and human serum with age. Silencing of MANF in fruit fly ubiquitously or specifically in immune cells (hemocytes) resulted in disrupted intestinal homeostasis, age-related activation of JAK/STAT signaling cascade in the intestine and decreased lifespan. Overexpression of MANF in the fat body, hemocytes, and gut enterocytes inhibited age-related inflammation, the loss of epithelial homeostasis and extended the lifespan of fruit flies. In contrast, MANF overexpression in neurons reduced the lifespan of fruit fly, suggesting distinct function of MANF in different cell types (Sousa-Victor et al., 2019). Deficiency of MANF in MANF heterozygote mice led to increased infiltration and activation of macrophages in white adipose tissue, pancreas, and liver already at 5 months of age and signs of cellular senescence at 10 months of age. MANF heterozygote mice displayed progressive liver dysfunction associated with activation of JNK signaling, increased hepatocyte apoptosis, signs of liver fibrosis and hepatosteatosis (Sousa-Victor et al., 2019).

Moreover, decreased levels of MANF in the blood was observed in humans with the liver disease as well as in the livers of wild type mice after a high-fat diet (HFD). Overexpression of human MANF in mice liver fed on HFD led to the reduced accumulation of fat and decreased the number of activated macrophages in the liver. Similarly to MANF heterozygote mice, specific ablation of MANF in immune cells also resulted in liver damage and inflammation, although no fat accumulation was detected. In contrast, specific ablation of MANF in hepatocytes did not lead to immune activation, liver damage, or fibrosis, but increased fat was observed in the livers of these animals. These results indicated that reduced expression of MANF associated with age contributes to disrupted immune and metabolic homeostasis.

In addition, MANF was identified as the factor responsible for rejuvenation (Sousa-Victor et al., 2019). Animals deficient in MANF did not promote rejuvenation in the old partner during heterochronic parabiosis. Hence, these data suggested that MANF is a conserved regulator of metabolic and immune homeostasis during aging in animals.

1.3.4 MANF therapeutic potential in ischemia models

Ischemia is a reason of increased incidence rate and death. The discrepancy between the blood supply and requirements in oxygen and nutrients within the ischemic organ results in the acute tissue hypoxia and microvascular dysfunction caused by a blood clot or atherosclerotic plaque (Eltzschig and Eckle, 2011). Reperfusion aiming to restore the blood flow and oxygenation is used first for the treatment of ischemia, although this process promotes the initiation of immune responses, oxidative stress and cell death programs. Novel treatment approaches are aiming to render tissues unaffected to ischemia or restrain reperfusion injury and are currently under investigation (Perricone and Vander Heide, 2014; George and Steinberg, 2015).

MANF has also been studied for its protective and restorative effects in rodent models of cerebral and myocardial ischemia (Airavaara et al., 2009; Airavaara et al., 2010; Glembotski et al., 2012; Yang et al., 2014b). Recombinant and overexpressed MANF effectively protected cardiomyocytes from death in simulated ischemia *in vitro* (Tadimalla et al., 2008). MANF protein subjected to the mouse model of myocardial ischemia resulted in reduced infarct zone compared to control mice (Glembotski et al., 2012). All three branches of the UPR are upregulated in heart damage after myocardial ischemia in rats (Zhang et al., 2017a), suggesting that the therapeutic potential of MANF in myocardial ischemia depend on its role in dampening ER stress.

Intracortical pretreatment with human MANF and virus vector-mediated MANF overexpression just before the cortical stroke model of middle cerebral artery occlusion (dMCAO) led to reduced infarct area and promoted neurological and behavioral recovery in rats (Airavaara et al., 2009; Airavaara et al., 2010). Similarly, treatment with MANF protein 2 h after dMCAO led to decreased infarct size and number of apoptotic cells assessed by the Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and cleaved caspase-3 staining compared to controls (Yang et al., 2014b). However, it was suggested that MANF exerted its beneficial effects by dampening chronic ER stress induced by ischemia (Yang et al., 2014b). Other studies revealed that virus vector-delivered MANF in the rat model of dMCAO led to functional recovery by the activation of innate immune system at the peri-infarct area surrounding cortical stroke (Matlik et al., 2018). Next the authors addressed the role of MANF in dMCAO model performed on the *NestinCre*$^{+/-}$::*Manf*$^{flox(fl)flox/(fl)}$ mice, where MANF was specifically ablated in the neurons of CNS (Matlik et al., 2018). The peri-infarct area in the brains of *NestinCre*$^{+/-}$:: *Manf*$^{fl/fl}$ mice were larger in comparison to the control *Manf*$^{fl/fl}$, revealing the protective properties of endogenous MANF in the mouse neurons. Notable, deficiency of MANF in the *NestinCre*$^{+/-}$:: *Manf*$^{fl/fl}$ stroke model did not alter the activation of immune cells, indicating differences between the functions of endogenous and overexpressed MANF. MANF was also shown to promote differentiation and migration of neural progenitor cells to the infarct area in the stroke cortex (Tseng et al., 2018). Thus, MANF could mediate faster recovery from stroke by reducing ER stress, through activation of the immune response and promoting tissue repair.

1.4 Genomic inactivation and overexpression of MANF in non-mammalian species

1.4.1 MANF knockout in the fruit fly

Previous studies addressed the biological roles of MANF *in vivo* in *Drosophila melanogaster*. Expression analysis revealed localization of DmMANF in glia and neurons of CNS and also in different peripheral tissues including salivary glands, fat tissue, trachea throughout the development of *D. melanogaster* and in adult ovaries (Palgi et al., 2009; Palgi et al., 2012; Stratoulias and Heino, 2015; Lindstrom et al., 2017). During embryo development, DmMANF is expressed only a subpopulation of glial cells in the nervous system (Palgi et al., 2009). However, DmMANF is expressed in both glia cells and neurons in the brain of adult *D. melanogaster* (Stratoulias and Heino, 2015).

Genomic inactivation of MANF in the fruit fly led to lethality at early larval stages due to defects in cuticle formation and the CNS with dramatic changes in dopamine levels and dopaminergic axonal degeneration, although the soma of dopamine neurons were intact (Palgi et al. 2009). Interestingly, the nervous system of *D. melanogaster* during embryogenesis looked normal due to the maternal contribution of MANF. However, flies, that lacked the maternal contribution, died during final stage of embryogenesis, showing a more severe CNS phenotype. However, ablation of DmMANF specifically in neurons of adult flies did not alter the dopamine system indicating that MANF is required during developmental stages and not for the maintenance of dopamine neurons (Stratoulias and Heino, 2015). The overexpression of DmMANF in a cell-autonomous manner did not lead to the differentiation of dopaminergic neurons (Stratoulias and Heino, 2015). Importantly, constrained expression of human MANF (not expressed in muscle, fat body or gastric caeca) could rescue the lethal phenotype of the MANF deficient *D. melanogaster*. The expression of C-terminal and N-terminal domains of MANF did not lead to protective effects when applied separately or even together, indicating the essential role of intact full-length MANF (Lindstrom et al., 2013). It should be noted, however, that the biological activity of MANF domains in fly was not analyzed in control experiments. Human CDNF was less potent than MANF and required ubiquitous expression to compensate for the loss of DmMANF (Lindstrom et al., 2013). Human CDNF was less potent than MANF and required ubiquities expression to compensate for the loss of DmMANF (Lindstrom et al., 2013).

Gene expression analysis of maternal-zygotic DmMANF-deficient embryos identified changes in the genes involved in perturbations in membrane transport and major metabolic changes (Palgi et al., 2012). The expression levels of genes related to stress, immune responses, proteolysis, and cell death were altered in MANF deficient *D. melanogaster*, as well as the genes for dopamine uptake, synthesis and transport were differentially regulated (Palgi et al., 2012). More than 40% of gene expression levels known as ER/UPR genes were differently regulated in DmMANF mutants. Moreover, increased phosphorylation of eIF2α was detected in DmMANF larvae indicating the activation of PKR-like ER kinase (PERK) pathway (Palgi et al., 2012), although slightly reduced levels of spliced form of X-box binding protein 1 (spXbp1) and no changes in GRP78 levels was observed (Lindstrom et al., 2016). These data indicated the association of MANF deficiency with activation of ER stress

and UPR. Specific ablation of DmMANF by RNA interference in glial cells led to acute deterioration only in the lamina epithelial glial cells and decreased the lifespan of mutants (Walkowicz et al., 2017). Additionally, the sleep and locomotor activity of flies were affected by reduced DmMANF in glia or neurons. Overexpression of DmMANF in different *Drosophila* lines led to an increase in genes associated with the damping the oxidative stress, suggesting roles for MANF in protecting the dopamine neurons from oxidative stress (Palgi et al., 2012).

Taken together, DmMANF is a vital factor for the development of *D. melanogaster*. Unfortunately, the role of MANF in the peripheral tissues of *Drosophila* was not addressed.

1.4.2 MANF knockdown in zebrafish

Silencing of MANF expression was also studied in zebrafish (*Danio rerio*) (Chen et al., 2012). Both MANF and CDNF genes are expressed in zebrafish. To date, CDNF function in zebrafish was not addressed. MANF is broadly expressed during embryo development and in the tissues of adult zebrafish. Knock-down of MANF protein during the development by antisense morpholino oligonucleotides in larval zebrafish did not lead to apparent phenotype and changes in viability. However, brain dopamine levels were reduced by half and the expression of the two genes for tyrosine hydroxylase, th1 and th2, encoding for the rate-limiting enzyme for dopamine synthesis, was reduced as well as number of TH-positive and dopamine transporter cells were decreased in specific brain areas. The changes on the dopamine level and the th1 / th2 expression could be partly restored by expression of exogenous zebrafish *Manf* mRNA.

1.4.3 MANF knockdown in *Caenorhabditis elegans*

A single ortholog of mammalian MANF/CDNF has been found in C.elegans. Ablation of MANF was studied in *Caenorhabditis elegans (C.elegans)* (Richman et al., 2018). Removal of the *manf-1* gene in *C.elegans* resulted in no apparent morphological defects, although slower growth was observed in MANF-deficient animals compared to wildtypes. The development of the dopaminergic, GABAergic and serotonergic neurons was normal in MANF mutants, indicating that MANF is not a key factor for the development and migration of neurons in *C. elegans*.

However, degeneration of somas of dopamine but not serotonergic or GABAergic neurons was observed in MANF mutants with enhanced age progression, implying a vital role for *manf-1* in neuroprotection of dopaminergic neurons.

MANF mutants displayed increased levels of inositol-requiring enzyme 1 (IRE1), Xbp1, and hsp4, a homolog of the human GRP78, indicating initiation of ER stress and UPR in MANF-deficient animals (Bai et al., 2018; Richman et al., 2018). No changes in hsp6 expression, an ortholog of the vertebrate heat shock proteins (HSP)70 mitochondrial matrix specific chaperone, was detected, implying that *manf-1* do not regulate mitochondrial UPR (Richman et al., 2018). The studies also revealed that human MANF could rescue the age-related degeneration of dopaminergic neurons *C. elegans manf-1* mutants (Bai et al., 2018).

Moreover, *manf-1* mutants exhibited behavioral defects, particularly slower swimming speed. On top of that, MANF-deficient *C.elegans* displayed that α-Synuclein aggregates were significantly enhanced, although it remains to be studied if *manf-1* has a direct role in this process (Richman et al., 2018). *manf-1* was shown to be broadly expressed in several major tissues, including intestine, hypoderm, spermatheca, and the nervous system in wild-type *C.elegans* (Bai et al., 2018). However, the expression levels of *manf-1* declines with age, which did not lead to the degeneration of dopaminergic neurons (Richman et al., 2018). Along with the growth delay, neurodegenerative phenotype and enhanced ER stress in *manf-1* mutants, this mutants had fewer offspring, indicating that MANF is required for the proper function of the reproductive system of *C.elegans*.

1.4.4 MANF knockdown and overexpression in the central nervous system of mice

In contrast to the neuronal phenotypes of MANF-deficient invertebrates and zebrafish, our studies revealed no major effect on the brain phenotype in adult conventional MANF knockout mice, where MANF was shown to play role in regulation of the neurite outgrowth during cortical development and neuronal migration, implying that MANF is required for cortical neurons migration in the developing mouse brain (Tseng et al., 2017). Moreover, the activation of UPR was found in neural stem cells (NSCs) isolated from E13.5 MANF-deficient embryos during differentiation, although no apoptosis was detected. Particularly, increased levels of *Grp78*, *spXbp1* and *Atf4* mRNA accompanied by the enhanced levels of phosphorylated eIF2α were observed at the 8th day of *in vitro* culture (Tseng et al., 2017), indicating the activation of PERK and IRE1cascades. Notably, the *Chop* mRNA levels were not altered in NSCs lacking MANF, suggesting that deficit of MANF in embryonic neurons does not alter cell survival mediated by CHOP.

Recent studies identified the role of MANF overexpression in the CNS neurons, where C-terminally tagged human MANF was expressed under the mouse prion promoter (Yang et al., 2017). MANF overexpression in the brain of transgenic mice revealed increased feeding behavior resulting in increased body weight associated with enhanced adipose tissues in 4-month-old mice fed with a regular chow diet. The same hyperphagic phenotype was found in mice which were induced to overexpress MANF specifically in the hypothalamus using MANF expressing adeno-associated virus vectors (AAV). Similarly to MANF-transgenic mice, the mice injected with AAV-MANF displayed increased body weights and hyperphagia 2 weeks after the injections. However, no alterations were observed in mice that received MANF protein into the third ventricle or hypothalamus. These results indicated that MANF functions intracellularly.

Moreover, MANF overexpression in the mouse hypothalamus led to altered insulin signaling but did not affect the leptin signaling. Enhanced expression of MANF was suggested to recruit phosphatidylinositol-5-phosphate 4-kinase type-2 beta (PIP4k2b) localization in the ER, where it becomes active reducing the phosphorylation of AKT, leading to impaired insulin signaling cascade and hyperphagia.

In accordance, either silencing MANF or neuron-specific deletion of MANF in the hypothalamus led to decreased food intake and consequent reduction in body weight due to increased AKT phosphorylation and insulin sensitivity (Yang et al., 2017).

1.4.5 The role of MANF-deficiency in skeletal tissue homeostasis

MED is a skeletal disease that is characterized by malformation of the cartilage and bones leading to moderate dwarfism, joint pain and early development of arthritis and associated with gene mutations in matrilin-3, cartilage oligomeric matrix protein and type IX collagen (Briggs and Chapman, 2002). The mutation in the gene encoding type X collagen gives rise to another type of skeletal disorder called MCDS, which is characterized by dwarfism accompanied by short-limbed dwarfism and bowed legs (Wallis et al., 1996). Mutation in matrilin-3 and type X collagen genes in mouse represents model of MED and MCDS respectively, which are associated with activation of ER stress and UPR in chondrocytes (Nundlall et al., 2010; Cameron et al., 2011). Previous studies revealed that *Manf* gene is highly upregulated in the mouse model of multiple epiphyseal dysplasias (MED) and metaphyseal chondrodysplasia type Schmid (MCDS) (Nundlall et al., 2010; Hartley et al., 2013).

MANF functions in skeletal tissue was addressed by the specific removal of MANF from cartilage (Bell et al., 2019). The cartilage-specific ablation of MANF result in a chondrodysplasia-like phenotype. The MANF conditional knockout mice had shorter long bones (tibia bone ↓5.4%, femur bone ↓3.5%) and reduced skull lengths (↓4.5%). However, the cartilage growth plate was morphologically normal. Deficiency of MANF led to decreased chondrocyte proliferation and dysregulated apoptosis. Transcriptomic analysis revealed increased in ER-resident proteins associated with ER stress and ER stress response marker GRP78. Deletion of *Manf* specifically from cartilage cells in a mouse model of multiple epiphyseal dysplasias (MED) led to the aggravation of the disease demonstrating further reduction in bone sizes and bell-shaped rib cages that impeded mice breathing (Bell et al., 2019). Hence, MANF is essential for chondrocyte ER homeostasis and bone growth.

2. ER stress and UPR

2.1 Overview of ER stress and UPR

ER is an important compartment that is required for protein synthesis, folding and transport. It is also an important site of Ca^{2+} storage and production of sterols and lipids (Ron and Walter, 2007). Initiation of ER stress and UPR is caused by the accumulation of unfolded and misfolded proteins in the ER, resulting in release of GRP78 and activation of intracellular signal transduction pathways, which are regulated by three ER transmembrane proteins - PERK, activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1) (Ron and Walter, 2007). The initiation of UPR is required in order to restore ER homeostasis (Ron and Walter, 2007). If ER stress remains unresolved the downstream consequences of UPR lead toward the inflammation, autophagy and apoptosis (Tabas and Ron, 2011).

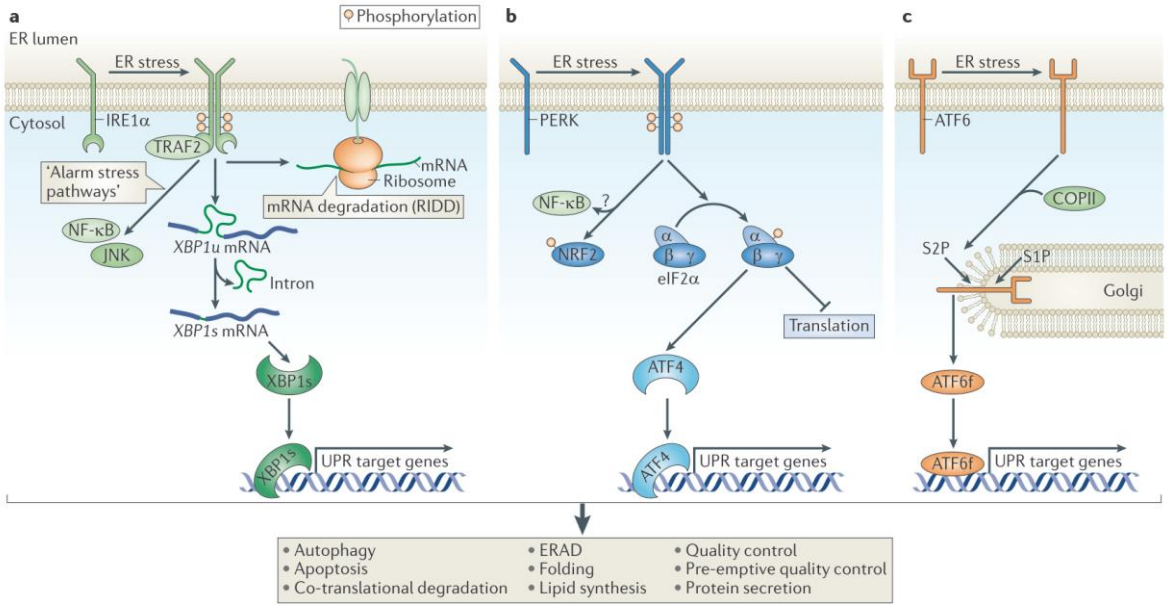

Figure 2. Schematic illustration of unfolded protein response (UPR) signaling pathways. UPR signaling pathways are mediated by PKR-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1), activation of which aim to restore folding of the proteins and increase degradation of the misfolded proteins thereby promoting cell survival. If ER stress remains unresolved, UPR cascades are directed to apoptotic signaling. Adapted from (Hetz, 2012).

IRE1 pathway is an evolutionarily conserved arm of UPR. In response to unfolded proteins, IRE1 dimerization and trans-autophosphorylation trigger its RNase activity, which initiates the increased splicing of *Xbp1* mRNA into a *spXbp1* encoding for an active transcription factor. spXbp1 translocates to the nucleus, where it activates the transcriptions of genes for ER chaperones, ER-associated degradation (ERAD) machinery, protein quality control and phospholipid biosynthesis. IRE1α also triggers the activation of regulated IRE1-dependent decay (RIDD) of ER-associated mRNAs, which reduces the ER workload for newly synthesized proteins (Pirot et al., 2007; Hollien et al., 2009). Additionally, oligomerized and chronically activated IRE1α targets the activation of pro-inflammatory and pro-apoptotic proteins. The RNase activity of IRE1α promotes translation of proapoptotic protease caspase-2 and pro-oxidant thioredoxin-interacting protein (TXNIP), thereby leading to activation of NLRP3 inflammasome and caspase-1 that cause maturation and secretion of IL-1β in pancreatic beta-cells (Lerner et al., 2012; Upton et al., 2012). Under chronic ER stress conditions, IRE1α binds to the scaffold protein tumor necrosis factor receptor-associated factor 2 (TRAF2) thereby resulting in activation of apoptosis signal-regulating kinase (ASK1) or caspase-12 at the ER membrane in various cell types (Urano et al., 2000; Yoneda et al., 2001; Nishitoh et al., 2002; Son et al., 2014). Finally, activation of IRE1α pathway triggers the initiation of pro-inflammatory pathways p38 MAPK and c-Jun N-terminal kinase (JNK) pathway via IRE1α/TRAF2/ASK1 complex and NF-κB signaling pathway via IRE1α/TRAF2 complex (Urano et al., 2000; Nishitoh et al., 2002; Kaneko et al., 2003; Kim et al., 2010). NF-κB stimulates the inducible isoform nitric oxide synthases (iNOS) subsequently leading to nitric oxide (NO) formation, that triggers cell death via several

mechanisms like oxidative stress, DNA damage via necrosis pathway through poly (ADP-ribose) polymerase (PARP) and via apoptosis pathway mediated through p53 signaling, or dysregulation of cytosolic calcium (Murphy, 1999).

The activation of PERK pathway leads to activation of the initiation factor eukaryotic translation initiator factor (eIF2α), phosphorylation of which is controlled by four unique kinases general control non-derepressible-2 kinase (GCN2), double-stranded RNA-activated protein kinase (PKR), PKR-like endoplasmic reticulum kinase (PERK), and heme-regulated inhibitor kinase (HRI) depending on stress response stimuli (Donnelly et al., 2013). Phosphorylation eIF2α leads to suppressed initiation of protein synthesis to relieve the ER load and increased translation of transcription factor ATF4 (Harding et al., 1999; Harding et al., 2000), which initiates the expression of ATF3 and CHOP and controls genes involved in apoptosis and autophagy (Harding et al., 2003; Schroder and Kaufman, 2006). CHOP activates the growth arrest and DNA damage protein (GADD34), leading to dephosphorylation of eIF2α via protein phosphatase 1 (PP1) and protein translation recovery in cells recovering from ER stress. During ER stress ATF3 potentiates the GADD34 expression, which contributes to feedback de-phosphorylation of eIF2α (Jiang et al., 2004). Transcriptional induction through ATF4 and CHOP enhances the pro-apoptotic protein production, depletion of ATP and formation of reactive oxygen species (ROS) resulting in oxidative stress and cell death (Han et al., 2013). Moreover, CHOP initiates cell death by constraining the anti-apoptotic regulators BCL-2 family and stimulating the expression of pseudokinase tribbles homolog 3 (TRIB3), an inhibitor of AKT (McCullough et al., 2001; Du et al., 2003; Marciniak et al., 2004). The trib3 expression is also regulated by ATF4 (Cunard, 2013). Additionally, up-regulation of the transcription factors ATF4, CHOP and TRIB3 leads to autophagy induction (Salazar et al., 2009). Hence, CHOP expression increases ER protein workload and consequently fosters ER stress and apoptotic cell death (Brush et al., 2003; Feldman et al., 2005). Additionally, phosphorylated eIF2α initiates the NF-κB cascade due to repression of its inhibitor (IκBα) translation (Deng et al., 2004). Finally, the initiation of PERK pathway leads to nuclear translocation of nuclear factor erythroid 2–related factor 2 (NRF2) that possesses the cytoprotective properties during ER stress (Cullinan et al., 2003; Ma, 2013; Mukaigasa et al., 2018).

Upon activation of ER stress, ATF6 is translocated to the Golgi, where it is cleaved by site 1 and 2 proteases, resulting in the release of its cytosolic domain fragment (ATF6f). ATF6f is a transcription factor that controls the expression of genes encoding the GRP78 and GRP94, spXbp1, CHOP, ERAD components, and genes implicated in regulation of lipid synthesis (Kaufman et al., 2010). ATF6α isoform carries functions of ER stress-induced gene expression subjected to rapid degradation, while ATF6β may negatively regulate ATF6α, acting as a feedback loop to regulate ATF6-dependent signaling (Thuerauf et al., 2007).

2.2 Dysregulated UPR in pathological states in human and animal models

The emerging evidence reveals that ER stress and UPR contributes to the pathophysiological and metabolic changes of many diseases in human and mouse models including diabetes, obesity, liver diseases, and neurodegenerative diseases (Kaufman, 2002; Hetz et al., 2019).

The association of ER stress and UPR with the disease pathology of diabetes and other metabolic disorders has been well documented and will be reviewed in detail in chapter 3.5. The function of UPR in the physiology of the nervous system is not well understood, although several indications have been proposed. Studies of Hayashi et al. revealed that Xbp1 is essential for BDNF-induced outgrowth via regulation of GABAergic markers (Hayashi et al., 2007; Hayashi et al., 2008). Genetic variants in the Xbp1 gene were associated with bipolar disorder and schizophrenia (Kakiuchi et al., 2003). Mutation in the PERK gene EIF2AK3 was found in a child with early signs of neurodegeneration (Bruch et al., 2015), indicating essential role for PERK in the neurons. Moreover, phosphorylated PERK and eIF2α was detected in the post mortem brain samples from patients with several neurodegenerative diseases including Alzheimer's disease, Parkinson's disease and sporadic amyotrophic lateral sclerosis (Hoozemans et al., 2007; Atkin et al., 2008; Hoozemans et al., 2009; Stutzbach et al., 2013). Interestingly, specific ablation of PERK in the neurons of 8 weeks old mice did not affect the viability of the neurons or axons under physiological conditions (Stone et al., 2019). However, PERK neuronal inactivation in the mouse model experimental autoimmune encephalomyelitis (EAE) exacerbated axon degeneration, neuron loss and demyelination (Stone et al., 2019). Neuron-specific ATF4 ablation did not aggravate the EAE. Studies on GCN2 knockout mice demonstrated that translational control of ATF4 is required for hippocampal synaptic plasticity, learning and memory (Costa-Mattioli et al., 2005). Reduced ATF4 expression in the mouse brain led to increased long-term synaptic plasticity and memory formation (Chen et al., 2003).

Neurodegenerative diseases differ in their clinical and pathological states but share a common sign – accumulation of misfolded disease-specific proteins associated with neuronal apoptosis. Alzheimer's disease is characterized by the aggregation of amyloid-beta and hyperphosphorylated tau, while the accumulation of α-synuclein was found the brains of human individuals with Parkinson's disease. The disease-specific TDP-43, FUS or SOD1 proteins are found in amyotrophic lateral sclerosis. However, it remains unclear if the accumulation of these disease-specific aglomerates triggers the activation of UPR or ER stress precedes these pathological state.

2.3 Regulation of MANF and CDNF expression by ER stress

Microarray studies revealed that MANF gene is upregulated in response to ER stress-induced by neurotoxins (Holtz and O'Malley, 2003). Other UPR inducers including tunicamycin (N-glycosylation inhibitor), thapsigargin (sarco/ER Ca^{2+} transporting ATPase inhibitor), lactacystin (proteasome inhibitor) and 1,4-Dithiothreitol (DTT, reducing agent) are also known to up-regulate MANF expression in cell lines and primary cells (Apostolou et al., 2008; Hellman et al., 2011; Glembotski et al., 2012; Henderson et al., 2013). Expression of MANF mRNA and protein is also enhanced under multiple pathophysiological conditions which are related to ER stress. *In vivo*, increased MANF expression along with initiation of ER stress was found in pancreatic beta-cells of diabetic Akita mice (Mizobuchi et al., 2007) and chondrocytes in genetic mouse models of chondrodysplasias (Hartley et al., 2013). Additionally, MANF expression was up-regulated in hypoxia-related conditions such as myocytes in a mouse model of heart ischemia (Tadimalla et al., 2008; Glembotski et al.,

2012) and in transient global and focal cerebral ischemia (Lindholm et al., 2008; Yu et al., 2010). Increased MANF expression was also detected in the developing mouse brain upon treatment with tunicamycin (Wang et al., 2015). Moreover, enhanced expression of MANF was observed in kidney cells after activation of ER stress (Kim et al., 2016; Kim et al., 2017). Moreover, MANF expression is dependent on XBP1- and ATF6-induced expression (Tadimalla et al., 2008; Wang et al., 2018), implying that MANF is ER stress-related protein. The mechanism whereby MANF expression is increased might depend on the ER stress responsive elements (ERSE) detected in the MANF promoter that are recognized by spXBP1 and ATF6, thus upregulated in the UPR (Lee et al., 2003; Mizobuchi et al., 2007; Tadimalla et al., 2008; Yang et al., 2014a; Wang et al., 2018). Moreover, MANF expression is dependent on XBP1- and ATF6-induced expression (Tadimalla et al., 2008; Wang et al., 2018), implying that MANF is ER stress-related protein.

The first indication that MANF is a secreted factor was provided in the work of Petrova et al. when MANF was discovered (Petrova et al., 2003). Further studies identified that unlike other ER stress-induced proteins, MANF could be secreted in stress-regulated conditions. Secretion of MANF was induced by thapsigargin (Glembotski et al., 2012; Henderson et al., 2013) and ischemia-reperfusion injury (Glembotski et al., 2012), but not tunicamycin, although the previous work of Tadimalla et al. showed MANF secretion from primary rat cardiac myocytes upon tunicamycin treatment (Tadimalla et al., 2008). Both thapsigargin and myocardial ischemia followed by reperfusion dysregulate calcium in the ER leading to initiation of ER stress and UPR, and MANF secretion that can be regulated via two possible mechanisms: interaction with GRP78 (Glembotski et al., 2012) and C-terminal RTDL ER retention motif of MANF that interacts with KDEL-receptors and promotes MANF secretion (chapter 1.1). Further studies revealed that MANF is also secreted to the urine after acute kidney injury after intraperitoneal tunicamycin injection or tubular ischemia/reperfusion (Kim et al., 2016). Moreover, MANF is also secreted in mouse models of chondrodysplasias (Hartley et al., 2013) and congenital nephrotic syndrome (Kim et al., 2016).

In contrast to MANF, there is less published data related CDNF responses to ER stress and UPR. CDNF expression has been shown to be up-regulated in response to tunicamycin-induced ER stress in primary rat hippocampal neurons and cultured rat myoblast cells (H9c2) *in vitro* (Liu et al., 2018; Zhang et al., 2018), although no effect was seen in studies with human bone osteosarcoma epithelial cell line (U2OS) (Apostolou et al., 2008). CDNF upregulation *in vivo* and increased secretion upon ER stress conditions has not been documented yet.

3. Diabetes mellitus

Diabetes mellitus (DM) is a heterogeneous group of metabolic disorders characterized by elevated blood glucose levels (hyperglycemia), caused by either insulin deficiency and/or insulin resistance. Common symptoms of DM are thirst, fatigue, polyuria, and changes in weight. Long-term complications of untreated diabetes include peripheral neuropathy, nephropathy, retinopathy, foot injuries, bone and joint problems, teeth and gum infections, cardiovascular disease and stroke, leading to massive morbidity and mortality. Based on the

estimations from the International Diabetes Foundation of 2017, DM affected 425 million people worldwide and is rapidly increasing with an estimation of 629 million people worldwide in 2045 (https://www.idf.org/aboutdiabetes/what-is-diabetes/facts-figures.html).

DM is classified into different forms: type 1 diabetes (T1D), type 2 diabetes (T2D) and monogenic diabetes. An estimated 10% of diabetes cases in the world are T1D, while the majority being T2D. Other types of diabetes account for roughly 1-5%.

3.1 Type 1 Diabetes

T1D is characterized by hyperglycemia due to the reduction of insulin secretion that is caused by the autoimmune attack of pancreatic beta-cells. The disease manifestation occurs when a significant number of the functional beta-cell mass is lost (40-80% depending on the strength of the adverse immune response, the age of disease onset, periods of remission (Weir and Bonner-Weir, 2013; Chen et al., 2017)) and individual's blood glucose begins to deviate (Figure 2). T1D is thought to be promoted by the combination of genetic, epigenetic and environmental factors. Several environmental risk factors were suggested to trigger the beta cell autoimmunity including viral infections (enteroviruses), intestinal microbiota, vaccinations, excess of hygiene, dietary factors, toxins and chemical compound in foods or water (Rewers and Ludvigsson, 2016).

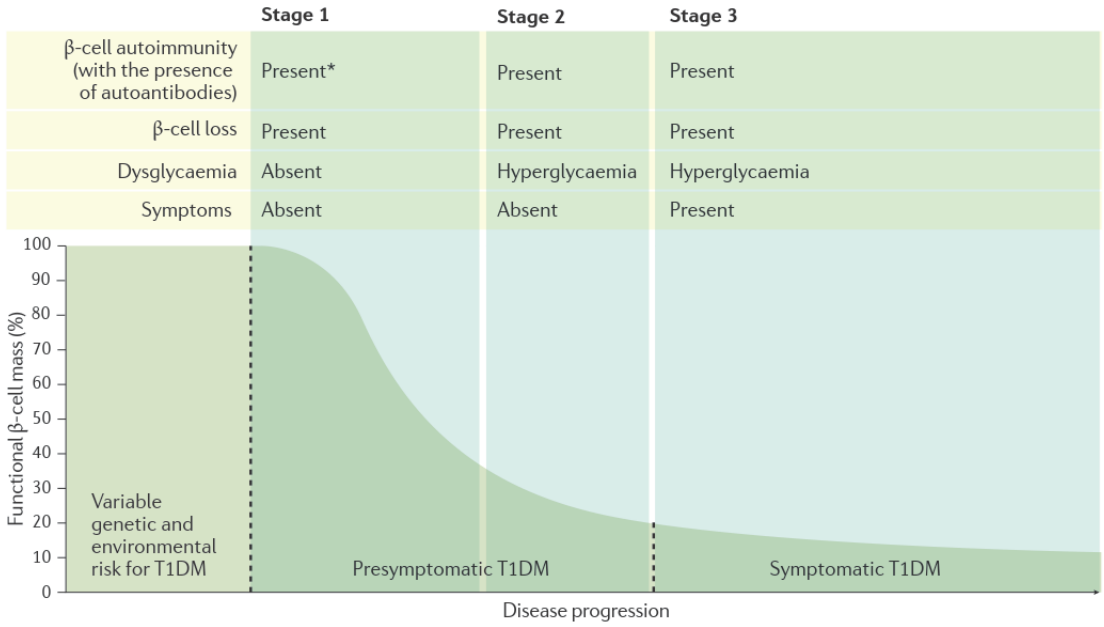

Figure 2. Suggested staging of type 1 diabetes (T1D). The pathogenesis of T1D is characterized by three stages, including stage 1, when no symptoms occur but beta-cell autoantibodies can be detected; stage 2, asymptomatic state, however functional beta-cell mass is gradually decreasing and biochemical tests may detect impaired glucose tolerance; stage 3, clinical onset of type 1 diabetes with its classic symptoms of polyuria, polydipsia and polyphagia, persistent hyperglycemia, remaining beta-cell generate and secrete insufficient insulin, beta-cell autoantibodies are still established, although some of them may have been lost. *beta-cell directed autoimmunity initiated by the beta-cell autoantibodies occuring months to years before the onset of the beta-cell loss. Adapted from (Katsarou et al., 2017).

T1D is defined as juvenile diabetes due to the age at the onset of the disease or insulin-dependent diabetes because insulin therapy is required for survival of the diagnosed individual. The evidence implies that beta-cell dysfunction and destruction begins after the onset of autoimmunity initiated by the beta-cell autoantibodies against different beta-cell proteins, which includes glutamate acid decarboxylase (GADA65a), insulin, zinc transporter 8 and insulinoma-associated antigen-2 (Couper et al., 2014). Roughly 70% of diabetic patients represent three or four autoantibodies, while only 10% have a single autoantibody. The duration from the appearance of autoantibodies until the clinical onset of diabetes might vary from a few months to decades.

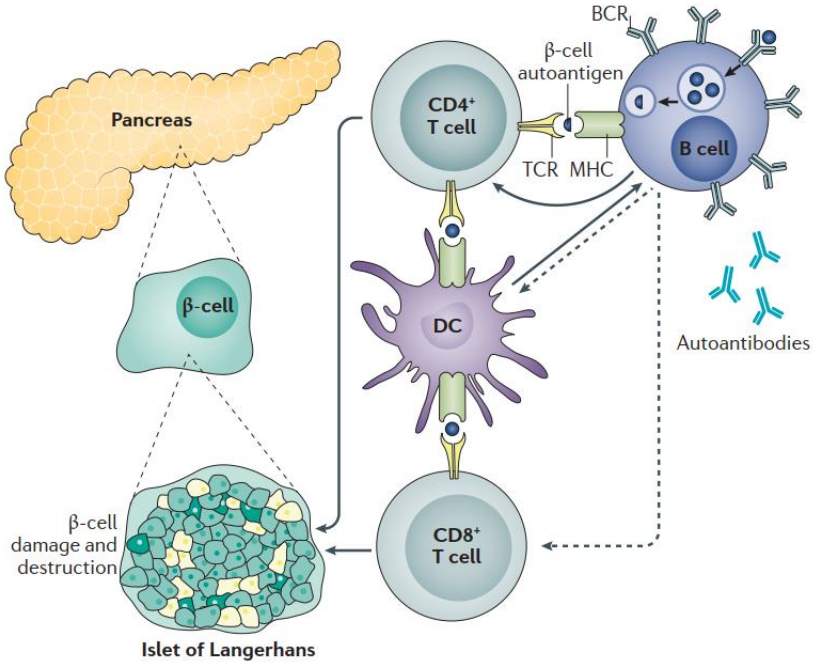

Figure 3. Type 1 diabetes mellitus (T1D) is an autoimmune disease. Antigen detection by B cells and dendritic cells (DCs) in the pancreatic islets triggers the activation of autoantigen-specific helper $CD4^+$ T cells and cytotoxic $CD8^+$ T cells. The activated T cells trigger the generation of pro-inflammatory cytokines, which contribute to the destruction of beta cells. The appearance of the beta-cell autoantigens results in generation of autoantibodies that target pancreatic islets and can be used as biomarkers of asymptomatic diabetes. Possible interactions between B cells and CD8+ T cells or DCs are marked with dashed arrows. BCR, B cell receptor; TCR, T cell receptor. Adapted from (Katsarou et al., 2017).

It is commonly accepted that beta cell destruction is triggered by the innate immune system, inflammation and activated autoreactive lymphocytes. The pathogenic pathway of the beta-cell destruction is not fully understood in humans, although some of the mechanisms were explained in mouse models such as non-obese diabetic mouse (NOD) and RIP–LCMV (rat insulin promoter-lymphocytic choriomeningitis) mouse models (Mathis et al., 2001; Lehuen et al., 2010). Cytotoxic T cells $CD8^+$, helper T cells $CD4^+$, natural killer cells, and macrophages promote damage of the beta-cells (Figure 3) (Lehuen et al., 2010). The activation of the innate immune system stimulates the release of proinflammatory cytokines

(Cabrera et al., 2016). The emerging evidence suggests that ER stress is involved in the pathogenesis of T1D and it will be reviewed in chapter 3.5 of this thesis.

Latent autoimmune diabetes in adults (LADA) is a rare form of autoimmune slowly progressing insulin-dependent diabetes that occurs in adults usually older than 30 years of age (Leslie et al., 2006). LADA was first defined in the early 1990s to characterize a group of patients who share the clinical and metabolic symptoms with both T1D and T2D (Tuomi et al., 1993). LADA is also referred to as 1.5 TD and diagnosed with three parameters based on Immunology of Diabetes Society: manifestation at the adult age (>30 years), the presence of diabetes-associated islet-targeting autoantibodies, and no need in insulin therapy at the onset of disease for at least 6 month (Juneja and Palmer, 1999; Fourlanos et al., 2005).

3.2 Type 2 Diabetes

T2D is a significant public health problem around the world. T2D is far more common compared to the other types of diabetes and is characterized by hyperglycemia that is caused by impaired insulin secretion and insulin resistance (DeFronzo et al., 2015). The etiology of T2D is highly associated with lifestyle factors accompanied by the genetic and environmental aspects. Obesity-related insulin resistance rises the incidence of T2D. The pathophysiology and progression of the T2D are variable among individuals. Some of the newly diagnosed patients are asymptomatic, whereas others have already severe hyperglycemia or even diabetic ketoacidosis (American Diabetes, 2009). Treatment of T2D includes changes in lifestyle (healthy diet, maintaining a body mass index of ≤25, physical exercising, avoiding smoking and consuming alcohol in moderation), and antidiabetic and anti-obesity medications (Hu et al., 2001; Schellenberg et al., 2013; Garvey et al., 2014). Upon treatment, severe hyperglycemia in T2D individuals can be reverted as a result of at least partial recovery of beta-cell function and decreased insulin resistance.

The beta-cell dysfunction in the presence of impaired insulin sensitivity is central to the pathology of T2D. Beta-cell increase insulin secretion to compensate for normoglycemia in obesity, and insulin-resistant states lead to enhanced beta cell function and expansion of the beta-cell mass (Prentki and Nolan, 2006). Several factors are known to trigger these compensatory effects such as increased nutrition (especially increased levels of glucose and free fatty acids (FFAs), growth factor signaling (insulin, placental lactogen, prolactin, etc.), and enhanced levels of and sensitivity to incretin hormones (glucagon-like peptide 1 (GLP-1)) (Prentki and Nolan, 2006; Auffret et al., 2013). It is still debated whether the islet expansion is caused by the proliferation of existing beta-cell and/or neogenesis of new beta-cells from pancreatic ductal cells (Bonner-Weir et al., 2010). If beta-cells fail to compensate for the enhanced demand, it will lead to beta-cell exhaustion, leading to reduced insulin secretion (Del Guerra et al., 2005). Several molecular mechanisms are involved in beta-cell de-differentiation and death in T2D including gluco- and lipotoxicity, mitochondrial dysfunction, oxidative stress, ER stress and inflammation (Alejandro et al., 2015). The implication of ER stress in the pathology of T2D will be discussed in the separate chapter 3.5.2.

Gestational diabetes mellitus (GDM) occurs during pregnancy in women and affects both the mother and unborn child throughout the pregnancy. Relatively high levels of human placental lactogen, leptin, prolactin, and cortisol from the placenta during the pregnancy may lead to the development of insulin resistance (Jao et al., 2013). GDM appears when beta-cells are not able to secrete enough insulin in the face of increasing insulin resistance (Cundy et al., 2014; McCabe and Perng, 2017). Women affected by GDM are at higher risk of developing metabolic syndrome and T2D over the years after their pregnancy, and their children have greater risk of developing obesity and T2D early in life (Group, 2002).

3.4 Monogenic diabetes

Monogenic diabetes or insulin-dependent form of DM is a rare type of DM that occurs due to beta-cell dysfunction and is caused by a single gene mutation essential for beta-cell development and regulation. It shares characteristics of both T1D or T2D and is therefore frequently misdiagnosed. There are two main classifications of monogenic diabetes: neonatal diabetes (NDM) and maturity-onset diabetes of the young (MODY). NDM manifests in the first 6 months of life and occurs rarely. Typically, newborns display intrauterine growth retardation and diabetes in the first week after the birth. If NDM develops a permanent condition, it is defined as permanent NDM (PNDM), while if it is presented transiently with apparent remission, it is called transient NDM (TNDM) (Murphy et al., 2008).

The most common mutations leading to PNDM are mutations in K_{ATP} channel genes and the proinsulin gene (INS). In more detail, mutations have been detected in K_{ATP} channel encoding genes KCNJ11 (encodes the inwardly rectifying potassium channel Kir6.2) and ABCC8 (encodes the sulfonylurea receptor [SUR] 1), leading to impaired insulin secretion and hyperglycemia (Gloyn et al., 2004; Flanagan et al., 2009).

To date, about 50 different mutations in the INS gene have been documented. These mutations impair the biosynthesis of insulin at a variety of different steps. Many of these mutations result in the misfolding of proinsulin with early manifestation of autosomal dominant diabetes, although some trigger diverse cellular and molecular mechanisms causing beta cell failure and diabetes. ER stress and UPR are triggered due to accumulation of misfolded proinsulin in the ER (Liu et al., 2005). The mutations in the INS gene are inherited in an autosomal dominant manner leading to PNDM, also known as Mutant INS-gene Diabetes of Youth (MIDY) (Liu et al., 2010).

Other rare cases of PNDM are not discussed in this thesis. Other gene mutations related to the activation of ER stress and UPR will be discussed in chapter 3.5.3.

MODY is diagnosed in young adults before the age of 25 years. The leading cases of MODY are caused by the mutations in the glucokinase (*GCK*) (MODY 2) and hepatocyte nuclear factor (*HNF)1A/4A* (MODY 3 and MODY 1) genes (Kim, 2015). *GCK* mutations cause an asymptomatic form of the disease with the mild and stable fasting hyperglycemia usually requiring no particular treatment. In contrary, *HNF1A* and *HNF4A* mutations result in progressive beta-cell dysfunction and elevated blood glucose levels that may lead to microvascular complications.

Hence, it is essential to precisely identify the genetic etiology of diabetes in order to select an appropriate treatment.

3.5 Chronic activation of unfolded protein response (UPR) as a cause of the beta-cell death

The beta-cells are continuously exposed to low levels of UPR activation as IRE1α and ATF6 signaling pathways are vital in normal ER biogenesis, required for differentiation of secretory cells (Calfon et al., 2002; Sriburi et al., 2007; Scheuner and Kaufman, 2008; Bommiasamy et al., 2009; Xin et al., 2018). However, high-stress conditions lead to beta-cell dedifferentiation, one of the mechanisms contributing for their dysfunction in diabetes (Akirav et al., 2008; Szabat et al., 2012; Weir et al., 2013; Brereton et al., 2014).

3.5.1 UPR in the pathogenesis of T1D

Chronic activation of the UPR and islet inflammation in T1D are highly connected (Brozzi and Eizirik, 2016). The mechanisms underlining the cytokine-induced beta-cell death are ER stress, ER calcium depletion, elevation of cytosolic calcium levels, and oxidative stress caused by ROS accumulation (Cardozo et al., 2005; Miani et al., 2012; Hara et al., 2014). Additionally, pro-inflammatory cytokines activate the JNK and NF-κB signaling cascades, leading to iNOS expression and NO formation followed by beta-cell death (Eizirik et al., 2009; Gurzov and Eizirik, 2011; Hasnain et al., 2016). However, the pathways behind cytokine-induced ER stress in beta-cells differ in humans and rodents. The exposure of beta-cells to cytokines results in SERCA2b inhibition (pump driving Ca^{2+} from cytosol into the ER), depleting ER Ca^{2+} (Cardozo et al., 2005). Activation of ER stress and UPR by pro-inflammatory cytokines in human and mouse beta-cells is independent of NO formation (Allagnat et al., 2012; Brozzi et al., 2015). By contrast, cytokine-induced NF-κB activation lead to NO production and initiation of the ER stress in beta-cells (Cardozo et al., 2005). Several studies showed that cytokine-induced activation of PERK and IRE1α/Xbp1 signaling pathways in beta-cells (Cardozo et al., 2005; Brozzi et al., 2015). Moreover, UPR by itself triggers the activation of inflammatory genes besides responding to inflammation. Chronic ER stress initiates the activation of several inflammatory pathways mediated by oligomerized IRE1α - JNK, p38 MAPK and NF-κB, which trigger beta-cell degeneration in diabetes (Brozzi and Eizirik, 2016).

The beta-cell dysfunction and death in diabetes are associated with the mitochondrial apoptotic pathway. Pro-apoptotic members of the Bcl-2 family, elevated levels of intracellular calcium, and ROS trigger cytochrome c release from mitochondria leading to beta-cell death (Gurzov and Eizirik, 2011). Pro- and anti-apoptotic proteins of Bcl2 family affect mitochondrial apoptotic pathway by regulating the permeabilization of the mitochondrial membrane by pore formation (Adams and Cory, 1998; Danial and Korsmeyer, 2004; Suhaili et al., 2017). Anti-apoptotic members such as Bcl-2, Bcl-XL and MCL-1 regulate cell survival by constraining the Bcl-2 pro-apoptotic proteins. The activation of pro-apoptotic members (Bad, Bim, Bid, Bcl10, Noxa, and Puma) affect the activity of anti-apoptotic molecules, consequently leading to the release of the pro-apoptotic effectors Bax

and Bak initiating the induction of mitochondrial permeability followed by release of cytochrome c and production of ROS (Danial and Korsmeyer, 2004; Strasser, 2005). Additionally, mitochondrial ROS production is enhanced upon increased cytosolic Ca^{2+} (Brookes et al., 2004), leading to ER Ca^{2+} release and beta-cell apoptosis mediated by cytochrome *c* via protease calpain-2 and caspases (Ramadan et al., 2011; Hara et al., 2014; Tsuchiya et al., 2016). Pro-inflammatory cytokines inhibit expression of pro-survival Bcl-2 proteins in pancreatic islets (Piro et al., 2001; Trincavelli et al., 2002; Van de Casteele et al., 2002) and Bcl-2 overexpression in beta-cells protects from cytokine-induced apoptosis *in vitro* (Rabinovitch et al., 1999; Barbu et al., 2002; Tran et al., 2003). Moreover, Bak/Bax was shown to increase RNase/kinase activity of IRE1α, followed by activation of XBP1 target genes and JNK pathway leading apoptosis (Hetz et al., 2006).

In T1D, it has been suggested that inflammation may lead to ER stress in beta-cells that in turn enhances beta-cell-inflammatory responses in several mouse models and human patients (Eizirik et al., 2008; Cnop et al., 2012). Inflamed islets from individuals with T1D display increased levels of ATF3, BiP/GRP78 and CHOP, although the expression levels of spXBP1 were not affected (Hartman et al., 2004; Marhfour et al., 2012). Importantly, CHOP knockdown led to decreased cytokine-induced beta-cell death and reduced activity of NF-κB signaling in primary human beta-cells and rat insulinoma-derived clonal (INS1-1E) cell line (Allagnat et al., 2012).

Based on the symptomatic and pathophysiological characteristics of T1D in humans, non-obese diabetic (NOD) mouse, which develops spontaneous auto-immune diabetes characterized by islet infiltration of inflammatory cells (insulitis), represent a mouse model of T1D (Anderson and Bluestone, 2005). Insulitis manifestation occurs in NOD mice by the age of 3 weeks and the onset of diabetes appears between the age of 12-20 weeks indicative for the decrease in beta-cell mass and overt hyperglycemia (Fujino-Kurihara et al., 1985). Several studies described the increased ER stress in the beta-cells of NOD mice contribute to the pathogenesis of diabetes. Tersey et al. identified increased ER stress and upregulation of UPR genes in the beta-cells before the development of diabetes in NOD mice (Tersey et al., 2012). Moreover, NF-κB signaling was also activated in the beta-cell of pre-diabetic NOD mice. Increased levels of *Grp78*, *spXbp1* and *Chop* mRNA were observed in the pre-diabetic NOD islets at the age of 6 weeks, while *Atf4* mRNA levels were decreased. Additionally, *Serca2b* mRNA was downregulated in 10 weeks old NOD islets, suggesting altered ER Ca^{2+} homeostasis. Similarly, in studies of Morita et al., islets of pre-diabetic mice showed increased mRNA levels of *Grp78* at 8 weeks of age, *spXbp1* at 8 and 10 weeks of age, and increased levels of auto-phosphorylated IRE1α at 10 and 12 weeks of age (Morita et al., 2017). Additionally, high levels of *Txnip* mRNA was detected in islets isolated from 12 weeks old NOD mice. Importantly, restraining the IRE1α activity by anti-oncogenic medication imatinib nearly fully reversed diabetes in NOD mice (Morita et al., 2017). The protective effects of imatinib were achieved by mitigating the interaction between the IRE1α and ABL tyrosine kinases found for its pro-apoptotic effects for beta-cells (Morita et al., 2017).

3.5.2 UPR in the pathogenesis of T2D

T2D is characterized by hyperglycemia due to insulin resistance in adipose tissue, muscles and liver, and/or decreased beta-cell insulin secretion, dysfunction, chronic ER stress and death due to chronic hyperglycemia and hyperlipidemia (Kahn et al., 2006; Cnop et al., 2010; Cnop et al., 2012).

Increased circulating free fatty acids (FFAs) due to over-nutrition and obesity results in beta-cell dysfunction and death in T2D (Prentki and Nolan, 2006). Both enhanced levels of saturated and unsaturated FFAs provoke UPR response in beta-cells by activating different signaling pathways (Cnop et al., 2010). Production of proinsulin and glucose-stimulated insulin secretion in beta-cells are inhibited by both unsaturated and saturated FFAs (Bollheimer et al., 1998). Saturated FFA, such as palmitate, led to activation of PERK (increased levels of ATF4, ATF3 and CHOP), IRE1α/Xbp1 (increased levels of spXbp1) and ATF6 pathways leading to beta-cell apoptosis (McGarry and Dobbins, 1999; Kharroubi et al., 2004; Cunha et al., 2008; Cnop et al., 2010). However, unsaturated FFAs, such as oleate, did not cause the activation of PERK and IRE1α signaling cascades, although it led to initiation of ATF6 pathways similarly to saturated FFAs (Cunha et al., 2008; Thorn and Bergsten, 2010). Additionally, saturated FFAs can initiate the UPR by perturbation of ER Ca^{2+} stores via diminishing the activity of SERCA (Back and Kaufman, 2012). Chronic hyperglycemia enhances the production of proinsulin and formation of islet amyloid polypeptide (IAPP) in the beta-cells (Haataja et al., 2008), following by increase in misfolded IAPP and ROS production. ROS and toxic IAPP disrupts ER Ca^{2+} homeostasis activates the UPR pathways leading to pro-apoptotic signals and proinsulin mRNA degradation (Hasnain et al., 2016). Moreover, IRE1α/Xbp1 pathway is activated in rodent beta-cells by acute and chronic glucose exposure *in vitro* (Lipson et al., 2006; Elouil et al., 2007).

Obesity is characterized by chronic inflammation with enhanced levels of pro-inflammatory cytokines and decreased levels of anti-inflammatory adipokines that originate from white adipose tissues (Pereira and Alvarez-Leite, 2014). In agreement with these observations, the enhanced levels of circulating pro-inflammatory cytokines were observed in early stages of T2D and increased gradually with the disease progression (Duncan et al., 2003; Grossmann et al., 2015). Similarly to the pathogenesis of cytokine-induced T1D, the circulating pro-inflammatory cytokines may induce ER stress and UPR in T2D islets. The increased levels of UPR markers such as Bip, Chop and TXNIP and decreased expression of IkBα and NRF2 were detected in the peripheral mononuclear cells in T2D patients (Cominacini et al., 2015; Lenin et al., 2015). However, it remains unclear whether beta-cell survival is affected by relatively low amounts of circulating cytokines. FFAs is another cause of pro-inflammatory responses through NF-κB signaling in the beta-cells (Eguchi et al., 2012; Pal et al., 2012). *In vitro* studies revealed that palmitate induces ER stress in human islets along with mild activation of NF-κB cascade and pro-inflammatory response, similar to T2D islets (Cunha et al., 2008). Hence, FFAs-induced inflammation promotes beta-cell-ER stress and disease progression.

In T2D, increased levels of UPR proteins Grp78, p58, activation of ATF3 and CHOP accompanied with distended ER was demonstrated in pancreatic islets (Hartman et al., 2004;

Marchetti et al., 2004; Laybutt et al., 2007). On the contrary, Engin et al. identified that islets of T2D patients display reduced levels of spXBP1, ATF6 and barely any phospho-eIF2α, suggesting that deficient beta-cell UPR leads to beta-cell death (Engin et al., 2014). The development of diabetes in leptin receptor-deficient db/db mice strongly resembles human T2D (Hummel et al., 1966; Coleman and Hummel, 1967; Coleman, 1978). The db/db (C57BL/KsJ) mouse develops hyperinsulinemia within 2 weeks of age and obesity by 3 to 4 weeks leading to hyperglycemia at age 8 weeks and beta-cell dysfunction (Dalboge et al., 2013). Enhanced beta-cell mass appear 10 weeks old animals with the decline in older animals at 34 weeks of age.

Importantly, impaired UPR signaling was observed in the insulin-resistant db/db mouse islets including enhanced expression of phosphor-eIF2α, increased splicing of *Xbp1* mRNA and expression of CHOP (Yusta et al., 2006; Laybutt et al., 2007). Similarly, significant upregulation in *BiP*, *Grp94* and *p58* mRNA levels and increase in pro-apoptotic *Atf3*, *Chop*, and *Trib3* were detected in the islets of prediabetic db/db mice at 6 weeks of age (Chan et al., 2013), revealing that ER stress is implemented in the development of T2D in db/db mice. Similarly to leptin receptor-deficient mice, leptin-deficient ob/ob mice is a model of obesity, insulin resistance and hyperinsulinemia at 3–4 weeks of age, with obesity evident by 4 weeks of age accompanied by hyperlipidemia (Lindstrom, 2007; Srinivasan and Ramarao, 2007). However, the ob/ob mouse model lacks complete beta-cell failure, indicating that diabetes is not acute (Coleman, 1978). Interestingly, enhanced expression of *Bip*, *Grp94*, *p58* and *spXbp1* mRNA was detected in the ob/ob islets, while the expression of pro-apoptotic *Atf3*, *Chop*, and *Trib3* was not changed (Chan et al., 2013). Thus the mild UPR could explain the lack of beta-cell failure in these mice.

Moreover, pathogenesis of T2D is associated with ER stress not only in pancreatic beta-cells but also in various metabolic tissues, such as hypothalamus, liver, adipose tissues and muscle, leading to the progression of T2D (Cnop et al., 2012). Chronic ER-stressed induced inflammation in metabolic tissues of obese individuals results in suppressed insulin receptor signaling (Gregor and Hotamisligil, 2011; Garg et al., 2012). UPR response in hypothalamus leads to activation of inflammatory responses, which cause leptin/insulin resistance during the obesity onset (Cnop et al., 2012). ER stress in the liver resulted in the development of steatosis and insulin resistance, and lipid metabolism in the liver is regulated by UPR. UPR response in adipose tissues causes inflammation and regulates adipokine secretion. Particularly, PERK and IRE1α pathways were found to be activated in the liver and adipose tissues of obese animals (Ozcan et al., 2004). Initiation of JNK pathway through IRE1α and TRAF2 was shown to cause insulin resistance in T2D (Aguirre et al., 2000; Hirosumi et al., 2002). Additionally, it was shown that saturated FFAs initiates the activation of UPR in the muscle (Cnop et al., 2012).

3.5.3 UPR in the pathogenesis of monogenic diabetes

The MIDY syndrome is a direct evidence of diabetes caused by the accumulation of misfolded proteins and ER stress, involving misfolding mutations in INS gene (Liu et al., 2015b). In accordance with MIDY syndrome, ER stress-associated beta-cell death was found

in the islets of Akita mouse model (heterozygous mice carry a mutated *Ins2* gene), which develop diabetes in early life (Yoshioka et al., 1997; Oyadomari et al., 2002b). The mutation in *Ins2* gene in Akita mouse leads to disturbed oxidative folding and trafficking of pro-insulin to the Golgi and secretory granules. Additionally, pancreatic beta-cell of Akita mouse demonstrated dilated ER, a hallmark for ER dysfunction, and a reduced number and size of secretory granules (Wang et al., 1999). As a consequence of these processes, *Grp78* and *Chop* mRNA were upregulated in the pancreases of Akita mouse leading to the beta-cell apoptosis (Oyadomari et al., 2002b). However, recent studies performed on Akita mouse model as well as newly developed induced pluripotent stem cells (iPSCs) from humans with insulin mutation C96R (the mutation in the same cysteine as in Akita mouse C96Y) revealed no apoptosis of the beta-cells in both models but decreased proliferation of the beta-cells associated with activation of ER stress and UPR, and diminished mTORC1 signalling (Balboa et al., 2018; Riahi et al., 2018).

Wolfram syndrome 1 (WS1) is a rare autosomal-recessive neurodegenerative disorder associated with the development of juvenile-onset diabetes mellitus, optic atrophy and deafness (Barrett and Bundey, 1997; Cryns et al., 2003). The pathogenesis of WS1 ascribed to the loss-of-function mutation in the *WFS*1 gene that encodes wolframin, a transmembrane ER protein, the expression of which is triggered upon ER stress (Fonseca et al., 2005). A common polymorphism in WFS1 predisposes to T2D (Sandhu et al., 2007), although some WFS1 variants result in T1D in Japanese (Karasik et al., 1989; Awata et al., 2000). Mutation in *Wfs1* gene in mice lead to the manifestation of diabetes due to activation of UPR, inhibition of intracellular calcium under glucose stimulation and disrupted cell cycle control (Riggs et al., 2005; Yamada et al., 2006). Wolframin 1 ablation in beta-cells leads to hyperphosphorylation of ATF6 and ER stress-induced beta-cell dysfunction and apoptosis (Fonseca et al., 2005). In line with the data from Wolfram syndrome postmortem pancreas, the increased expression of *CHOP*, *ATF4*, *GRP78*, and *spXBP1* were observed in the beta-cell deficient in Wolframin (Ishihara et al., 2004; Fonseca et al., 2010; Shang et al., 2014). Additionally, WS1 human induced pluripotent stem cells-derived neural progenitor cells and wolframin ablation in mice display impaired ER Ca^{2+} homeostasis, followed by initiation of the cysteine protease calpain and cell death (Lu et al., 2014).

Wolcott-Rallison syndrome (WRS) is a rare genetic disease that caused by recessive homozygote mutations in the gene encoding eIF2α kinase 3 (EIF2AK3), known as PERK, and associated with neonatal insulin-deficient diabetes, defects in exocrine pancreas, hepatic steatosis, microcephaly, intellectual disability, skeletal dysplasia and growth retardation (Wolcott and Rallison, 1972; Stoss et al., 1982; Delepine et al., 2000; Senee et al., 2004). Likewise, embryonic inactivation of the *Perk* gene (*Perk*$^{-/-}$) in mice leads to the loss of insulin-producing beta-cells and diabetes that is accompanied by failure of the exocrine pancreas, skeletal dysplasias in newborns and postnatal dwarfism, including deficient bone mineralization, osteoporosis, and abnormal compact bone development (Harding et al., 2001; Zhang et al., 2002). Pancreatic beta-cells in *Perk*$^{-/-}$ mice display ER distention, high rate of beta- cell apoptosis and decreased beta-cell proliferation. Additionally, these mice display excessive proinsulin synthesis (Harding et al., 2001), which is associated with increased proinsulin misfolding (Liu et al., 2005), suggesting ER overload and unresolved UPR

response leading to apoptosis. Studies of Gao et al. revealed that PERK ablation in the pancreases of either young adult or aged mice led to the development of diabetes associated with loss of islet beta-cells, indicating that PERK is required for maintaining glucose homeostasis in adult pancreas (Gao et al., 2012). PERK ablation resulted in ER distention and the accumulation of proinsulin and glucose transporter 2 (GLUT2) intracellularly. Moreover, PERK excision triggered the increased levels of GRP78 (both protein and mRNA) and *Grp94* mRNA, and activation of two other remaining UPR branches – IRE1α and ATF6 cascades and their downstream targets Xbp1 and JNK, leading to initiation of pro-apoptotic signaling cascade, while no increase in phosphorylated eIF2α was detected in PERK-excised islets (Gao et al., 2012). Taken together, PERK-mediated phosphorylation of eIF2α is essential for modulating proinsulin synthesis, folding and trafficking, and for ER quality control in pancreatic beta-cells (Gupta et al., 2009; Harding et al., 2012).

In accordance with these observations, homozygous mice with a knock-in mutation in eIF2α (Ser51Ala) were lethal at neonatal stage with weak survival of the embryonic beta-cell (Scheuner et al., 2001). The mutation in Ser51Ala blocks stress-induced eIF2α translation attenuation and ATF4 transcriptional activity. Mice with the heterozygous state of eIF2α fed with the HFD displayed insulin resistance and functional alterations in beta-cells, although no signs of beta-cell failure were detected when mice were on the regular diet (Scheuner et al., 2005). Beta-cell dysfunction occurred as a result of misfolded proinsulin accumulation and ER dilation.

$P58^{IPK}$ (DNAJC3) is ER stress-inducible Grp78 co-chaperon, which represses PERK pathway by inhibiting the activation of eIF2α signaling (Yan et al., 2002; van Huizen et al., 2003). DNAJC3 loss-of-function mutations in humans associated with diabetes and several neurodegenerative aspects, including ataxia, upper-motor-neuron damage, peripheral neuropathy, hearing loss and cerebral atrophy (Synofzik et al., 2014). Ablation of $p58^{IPK}$ in mice results in the development of diabetes, characterized by beta-cell apoptosis and insulin deficiency associated with promoting of the PERK-CHOP signaling cascade (Ladiges et al., 2005; Oyadomari et al., 2006; Laybutt et al., 2007). Interestingly, ablation of the *Atf4* gene in mice did not affect either beta-cell function or survival, although diabetic $Perk^{-/-}$ mice displayed revoked ATF4 signals (Back et al., 2009). However, ATF4-deficient mice display abnormal bone formation, consequently leading to decreased bone mass (Yu et al., 2013), indicating that ATF4 is required for the skeletal homeostasis.

Mice deficient in IRE1α develop widespread abnormalities, resulting in death after 12.5 days of embryo development (Zhang et al., 2005). Specific ablation of IRE1α in the beta-cells in mice during embryo development as well as deficiency of IRE1α particularly in the beta-cells of adult mice resulted in the manifestation of diabetes due to altered function of the beta-cells (Hassler et al., 2015; Tsuchiya et al., 2018). Ablation of IRE1α from both mouse beta-cells and hypothalamus led to obesity and insulin resistance with more severe phenotype than in control mice after exposure to HFD (Xu et al., 2014). These mice displayed reduced beta-cell mass caused by decreased beta-cell proliferation, possibly due to a decrease in spXBP1-dependent expression of cyclin D1 (Xu et al., 2014). Deficiency of Xbp1 particularly in the beta-cells led to reduced proinsulin processing, insulin secretion, and beta-cell proliferation as a result of IRE1α hyperactivation (Lee et al., 2011). Mice heterozygous

to XBP1 developed impaired glucose tolerance and disrupted insulin signaling, when subjected to high fed diet, due to activation of PERK and JNK signaling (Ozcan et al., 2004).

In accordance with the phenotype of IRE1α knockout mice, deficiency of both ATF6α and ATF6β in mice causes lethality by 8.5 days of embryo development, although a single knockout of each gene does not cause developmental defects (Wu et al., 2007; Yamamoto et al., 2007). Later it was demonstrated that Atf6α-deficient mice display decreased insulin secretion under the conditions of HFD or when carrying the Akita Ins2 allele (Usui et al., 2012). Specific ablation ATF6α in the beta-cells has no effect on its development and function (Engin et al., 2013). Human patients diagnosed with achromatopsia, a cone photoreceptor defect, are carriers of Atf6α "hypomorphic" mutations (Kohl et al., 2015). In contrast to PERK signaling, to date, there is no evidence of the implication of IRE1α or ATF6 signaling pathways in human monogenic diabetes.

Table 1. Summary of the phenotypes in mice, lacking UPR genes. Modified from (Hetz, 2012).

Phenotype	**IRE1α**	**Xbp1**	**Atf6α**	**PERK**	**ATF4**
Full knockout					
Embryonic lethal	Yes	Yes	No*	No	No
Postnatal death	-	-	No	Yes	No
Tissue specific effects					
Endocrine pancreas and insulin secretion alteration	Yes	Yes	Yes**	Yes	No
Altered bond formation	-	-	-	Yes	Yes
Impaired glucose metabolism	Yes	Yes	-	Yes	No

* - double knockout of ATF6α and ATF6β in mice causes embryonic lethality by 8.5 days of gestation (Wu et al., 2007; Yamamoto et al., 2007), ** - Atf6α-deficient mice display decreased insulin secretion under the conditions of HFD or when carrying the Akita Ins2 allele (Usui et al., 2012).

3.6 Current and future therapies for the treatment of diabetes

Currently, there is no cure for T1D and T2D and the treatment of diabetes depends on its pathophysiology and tailored towards preventing or delaying the manifestation of late disease complications, decrease death incidence, and preserve a good quality of life. In the case of T1D, the current treatment is the administration of insulin or insulin analogs, which has significantly reduced death rates associated with DM and its complications. Insulin injections could expose to a higher risk, including hypoglycemia and hyperglycemia periods, as well as causing distress to patients. Marked clinical success was also achieved with pancreatic islets transplantation from cadaveric donors as another possibility for T1D therapy (Bruni et al., 2014). However, availability/selection of the donor and immunosuppression side effects persist as current complications of this therapy, as well as, recurrence of the disease. Human pluripotent stem cells are introduced as an alternative source of the pancreatic endocrine beta-cells (Sneddon et al., 2018). Clinical trials (1/2 phase) are ongoing by Viacyte company aiming to treat T1D with human stem cell-derived islet cell implants (https://viacyte.com/archives/press-releases/center-for-beta-cell-therapy-in-diabetes-and-viacyte-announce-start-of-european-clinical-trial-of-human-stem-cell-derived-implants-in-

type-1-diabetes-patients). The choice of therapies for other types of diabetes include different antidiabetic drugs: 1) sulphonylureas and insulin secretagogues used in some PNDM, MODY and T2D (Kalra et al., 2018); 2) metformin for T2D (Song, 2016); 3) incretin mimetics, modified GLP-1 analogs like Exendin-4, Liraglutide and Exenatide LAR for T2D (Gupta, 2013) and others. However, antidiabetic medications can lead to many potential side effects including hypoglycemia, nausea, upset stomach, weight gain, risk of liver disease, kidney complications and other. Insulin therapy may also be required for the treatment of T2D and monogenic forms of diabetes due to beta-cell death and insulin deficiency occurring with the disease progression.

Regeneration of the beta-cell mass is thus a potential cure for insulin-dependent diabetes and under extensive investigation. Restoring the functional beta-cell mass in diabetes could be achieved by 1) enhancement of beta-cell replication (Dor et al., 2004), 2) promoting neogenesis of the beta-cell from progenitors within the pancreas (Xu et al., 2008) and 3) transdifferentiation of different pancreatic islet cells to functional beta cells (Thorel et al., 2010). Several growth factors are known to regulate beta-cell proliferation (Table 2). Glucose infusion itself acts as a mitogen for beta-cells in rodents (Bernard et al., 1998; Alonso et al., 2007).

Table 2. Summary of the major growth factors that are known to stimulate beta-cell proliferation.

Growth factor	Effects on beta-cell proliferation	Reference
Placental lactogen	*in vitro*: ↑ beta-cell proliferation *in vivo*: *RipCre::PL1*, ↑ beta-cell mass, ↑ beta-cell proliferation	(Brelje et al., 1993; Vasavada et al., 2000; Cozar-Castellano et al., 2006)
Prolactin	*in vitro*: ↑ beta-cell proliferation *in vivo*: $PrlR^{-/-}$, ↓ beta-cell mass	(Brelje et al., 1993; Freemark et al., 2002)
Growth hormone	*in vitro*: ↑ beta-cell proliferation *in vivo*: $GHR^{-/-}$, ↓ beta-cell mass, ↓ beta-cell proliferation	(Brelje et al., 1993; Liu et al., 2004)
GLP-1 analogs (Exendin-4)	*in vitro*: ↑ beta-cell proliferation *in vivo*: *GLP1-Receptor*$^{-/-}$, no effect on beta-cell mass, Exendin-4 treatment, ↑ beta-cell proliferation, ↑ beta-cell mass	(Scrocchi et al., 1996; Xu et al., 1999; Ling et al., 2001; Song et al., 2008)
Insulin, Insulin-like growth factors	*in vitro*: ↑ beta-cell proliferation *in vivo*: *Rip-Cre::IR*$^{fl/fl}$, ↓ beta-cell mass, *RipCre::IGF-I Tg*, ↑ beta-cell proliferation, no effect on beta-cell mass, beta-cell apoptosis and neogenesis *Rip-IGF-II Tg*, ↑ beta-cell mass *IGF-II Tg*, ↑ beta-cell proliferation, ↑ beta-cell mass, ↓ beta-cell apoptosis *Pdx1Cre::IGF-I*$^{fl/fl}$, ↑ beta-cell mass	(Petrik et al., 1999; George et al., 2002; Paris et al., 2003; Lu et al., 2004; Otani et al., 2004)
Epidermal growth factor (EGF)	*in vitro*: modest effect *in vivo*: *HIP-EGF Tg*, ↑ beta-cell mass, ↑ beta-cell proliferation $EGF^{-/-}$, no effect of beta-cell mass	(Krakowski et al., 1999; Luetteke et al., 1999; Bernal-Mizrachi et al., 2014)
Platelet-derived growth factor (PDGF)	*in vitro*: PDGF-AA, ↑ beta-cell proliferation *in vivo*: *RIPCre:: PDGFRa*$^{fl/fl}$, ↓ beta-cell mass	(Chen et al., 2011)
Hepatocyte growth factor (HGF)	*in vitro*: ↑ beta-cell proliferation *in vivo*: Rip-HGF Tg, ↑ beta-cell mass, ↑ beta-cell proliferation	(Otonkoski et al., 1996; Garcia-Ocana et al., 2000; Garcia-Ocana et al., 2001; Cozar-Castellano et al., 2006)

While several studies do not show neogenesis in adult mice either physiologically or after pancreatic duct ligation (Solar et al., 2009; Xiao et al., 2013), other studies identified pancreatic adult stem/progenitor cells *in vivo* (Xu et al., 2008). Duct cells were shown to promote differentiation of endocrine cells after the diphtheria toxin-induced cell death expressed under the Pdx1 promoter and after pancreatic duct ligation in mice and a partial pancreatectomy in rats (Criscimanna et al., 2011; Bonner-Weir et al., 2012). Islet neogenesis associated protein-pentadecapeptide (INGAPPP) were shown to promote neogenesis and

reverse streptozotocin-induced diabetes in mice (Bonner-Weir et al., 1993; Gu and Sarvetnick, 1993). Moreover, the overexpression of transforming growth factor-α (TGF-α) induced the expansion of Pdx1-expressing ductal cells, increasing islet neogenesis (Song et al., 1999).

Several studies reported transdifferentiation of alpha- to beta-cells in response to pancreatic injury (Chung et al., 2010; Thorel et al., 2010). Conversion of alpha-cell to beta-cells was also demonstrated under genetic reprogramming (Collombat et al., 2009). The transgenic expression of *Pdx1* in *Ngn3* positive cells and the expression of *Pax4* in alpha-cells activated the regeneration of functional beta-cell mass by conversion of alpha-cells into beta-cells and protected from streptozotocin-induced diabetes in mice (Collombat et al., 2009). Recent studies confirmed the transdifferentiation of alpha-cells to beta cells in a transgenic zebrafish model of beta cell ablation (Ye et al., 2015). Importantly, knockdown of the *glucagon* gene led to diminished regeneration of the beta-cells, indicating that *glucagon* is required for alpha-to-beta cell transdifferentiation (Ye et al., 2015). Studies of Chera et al. demonstrated that alpha-to-beta cell fate switching occurs from puberty through adulthood, and also in aged individuals (Chera et al., 2014). However, before puberty, the transdifferentiation arises form somatostatin-producing delta-cells, when the alpha-to-beta cell conversion is not active (Chera et al., 2014).

Emerging evidence indicates that beta-cell regeneration can be implemented by targeting ER stress and UPR in diabetes. Several chemical compounds and drugs were demonstrated to target UPR by 1) direct interaction with the components of the UPR, 2) reduction of ER stress, 3) suppression of protein degradation, 4) promoting antioxidant activity and 5) regulation of ER calcium signaling (Hetz et al., 2013). Chemical chaperones such as 4-phenyl butyric acid (PBA) and taurine-conjugated ursodeoxycholic acid (TUDCA) was shown to be effective in targeting the UPR and alleviating ER stress in various animal models. PBA and TUDCA restored glucose metabolism and insulin sensitivity in ob/ob mice by diminishing the activity of PERK and IRE1α/JNK signaling, indicating their potential use for the treatment of T2D (Ozcan et al., 2006).

Studies with the NOD mice also revealed beneficial effect of TUDCA, including reduced insulitis, decreased beta-cell death, improved insulin secretion and normalized expression of ATF6 and Xbp1(Engin et al., 2013). Furthermore, TUDCA is underway in a clinical trial of recent-onset T1D (https://clinicaltrials.gov/ct2/show/NCT02218619). Oral administration of PBA to humans was suggested to reduce insulin resistance in T2D patients (Xiao et al., 2011).

Several other small molecules that diminish the activity of UPR in diabetic models have been identified. The ability of Exendin-4 to protect from ER stress-induced beta-cells apoptosis *in vitro* were shown in several studies (Yusta et al., 2006; Cunha et al., 2009; Oh et al., 2013). Additionally, Exendin-4 reverse ER stress-mediated beta-cell death in diabetic Akita and db/db mouse model (Yusta et al., 2006; Yamane et al., 2011). Therefore, Exendin-4 is a potential treatment for T1D and other monogenic types of diabetes associated with ER stress.

Morita and colleagues identified that targeting the interaction of cytosolic ABL kinases with IRE1α could be a potential target for the treatment of autoimmune diabetes (Morita et

al., 2017). The ABL-IRE1α axis was shown to potentiate apoptosis during ER stress and anti-cancer drug imatinib was able to decrease beta-cell death by diminishing the interaction of ABL with IRE1α and preventing the proapoptotic UPR thereby reversing diabetes in NOD mice (Morita et al., 2017). Moreover, KIRAs that selectively inhibit kinase/RNase activity of IRE1α promoted recovery of the beta-cells in the NOD and diabetic Akita mouse models (Morita et al., 2017). Thus, current study holds promise for imatinib and KIRAs to be used for the treatment of diabetes associated with ER stress.

AIMS OF THE STUDY

This study was designed to elucidate the biological functions of MANF *in vivo* by careful characterization of MANF conventional and conditional knockout mice phenotypes.

The specific aims were:

- To study in detail the expression of MANF in mouse tissues involved in metabolic homeostasis
- To study CDNF expression in mouse tissues
- To characterize the phenotypes of MANF-deficient mice
- To study the signaling pathways affected by MANF deficiency in pancreatic beta-cells
- To investigate the exogenous effect of MANF protein on mouse pancreatic beta-cells *in vitro*
- To assess the effect of MANF-overexpression in mouse beta-cells on experimentally induced T1D diabetes *in vivo*
- To implement a new fast and fully automated graphical software for the histological analysis of mouse pancreas based on the Deep Convolutional Neural Networks technique

MATERIALS AND METHODS

The main methods used by the thesis author are presented in the Table 3. Detailed descriptions of the materials and methods can be found in the original publications and manuscripts and their supplements.

Table 3. Methods used in the studies.

Methods	Used in	The author contributed to the experiments
Mice and physiological tests *in vivo*		
Generation of *Manf*$^{-/-}$ and *Manf*$^{fl/fl}$ mice	II, III	
Mice genotyping	I, II, III	+
Glucose tolerance test, insulin tolerance test, and glucose challenge test followed by analysis of blood samples	II, III	+
Comprehensive Laboratory Animal Monitoring System (CLAMS)	II	
Molecular biology experiments		
DNA, RNA and protein isolation	I, II, III	+
cDNA synthesis	I, II, III	+
Real Time PCR and Quantitative PCR	I, II, III	+
Immunological methods		
Western blotting analysis	II, III	+
Immunohistochemistry	I, II, III, IV	+
Pancreatic islet isolation	II, III	+
Enzyme-linked immunosorbent assay (ELISA)		
Insulin ELISA from sera and tissue culture media	II, III	+
Mouse MANF ELISA from tissue	I	+
Mouse CDNF ELISA from tissue	I	+
Cell culture experiments		
Pancreatic islet isolation	II, III	+
Cell lines and primary cell culture	II, III	+
In vitro experiments with primary cells	II, III	+
In vitro insulin release experiments	II, III	+
Cytospins	II, III	+
Immunocytochemistry	III	+
Subcellular localization analysis	III	+
Animal models		
Multiple low-dose streptozotocin-induced mouse model of diabetes type 1	II	+
Pancreatic intraductal delivery of AAV6-MANF	II	+
Imaging		
One-photon microscopy imaging	I, II, III	+
3D HISTECH Panoramic 250 FLASH II digital slide scanner	I, III, IV	
Confocal microscopy	III	+
Electron microscopy	III	
Quantitative image analysis	I, II, III, IV	+
Statistical analysis	I, II, III, IV	+

Unpublished methods

MIN6 cell culture and immunocytochemistry

Mouse insulinoma cell line (MIN6) was cultured in the Dulbecco's modified Eagle's medium (DMEM, Sigma) media supplemented with 10% fetal bovine serum, 70μM β-mercaptoethanol and antibiotics in humidified 5% CO_2 at 37°C. For immunocytochemistry, MIN6 cells were cultured on the coverslips plated with poly-L-lysine and fixed in 4% paraformaldehyde for 15 minutes at room temperature. The cells were stained with antibodies to MANF (1:1000, 310-100, Icosagen), insulin (1:200, ab7842, Abcam), GRP78 (1:500, sc-1051, Santa Cruz Biotechnology), PDI (1:200, ADI-SPP-891-F, Enzo/AH Diagnostics) and GM130 (1:200, 610823, BD Transduction Laboratories), following by the labeling with Alexa Fluor® 488 or 568 secondary antibodies (1:400, Molecular Probes, Life Technologies) and DAPI (Vectorshield, Vector laboratories).

RESULTS AND DISCUSSION

4. MANF and CDNF expression in mouse tissues

Neurotrophic factors are growth factors that not only promote the survival and regeneration of the neurons but often also act outside the nervous system. MANF and CDNF family of the growth factors differ structurally and functionally from the other classical neurotrophic factor families. The protective and restorative properties of MANF and CDNF have been documented for both neuronal and non-neuronal cells. A better understanding of MANF and CDNF tissue distributions could provide important clues to their roles in CNS and peripheral target tissues, which in turn could be utilized to discover the most promising indications for these growth factors. Expression of MANF and CDNF was previously characterized (chapter 1.2). However, a full-scale report on the analysis of MANF and CDNF expression pattern in mouse embryonic and adult tissues have not been published. Therefore, an expression study using different approaches like IHC, QPCR and in-house build ELISA was justified. The comparison of antibody immunoreactivity in wild-type tissues with knockout animals, where the protein of interest has been silenced by genetic modification, is the most stringent control for antibody specificity. To ensure antibody sensitivity and specificity in our studies, we validated MANF and CDNF antibodies by using *Manf* and *Cdnf* knockout tissue as negative controls.

4.1 MANF expression during embryo development

(Original publication **I** and **III**)

We studied MANF expression in mouse embryos at different developmental stages by LacZ staining of heterozygous *Manf*$^{+/-}$ embryos expressing the β-galactosidase reporter gene under the *Manf* promoter and by IHC with MANF antibodies of wild-type (WT) *Manf*$^{+/+}$ embryos. In the current study, we demonstrated by LacZ staining for the first time that MANF is expressed already in the embryonic stem cells (Supplementary Figure 1A, **I**). At E7.5 LacZ positive staining was detected in all germ layers, revealing that MANF is important for the development of various tissues derived from all three germ layers. Similarly to LacZ staining, immunohistochemical analysis revealed MANF immunoreactivity in the developing E9.5 mouse embryo (Figure 1E-H, **I**). MANF expression was observed in the forebrain, midbrain and hindbrain parts of the neural tube. Additionally, MANF positive expression was found in the somites (Figure 1G, **I**), structures that are needed for the development of the dermis, skeletal muscles, and vertebrae. We also detected MANF immuno-reactive cells in the developing heart (Figure 1E, **I**).

Consistent with the findings of ISH done on E12.5 mouse embryos (Lindholm et al., 2008), we observed widespread expression of MANF at E13.5 in mouse embryos (Figure 1I-Y, Supplementary Figure1E-J, **I**), when most of the organs begin to differentiate. MANF expression was observed in the neocortex (Figure 1K, **I**), striatum (Figure 1M, **I**), midbrain (Figure 1N, **I**), developing hypothalamus parts (Figure 1O-P, **I**), spinal cord, choroid plexus (Figure 1L, **I**), the lumen of Rathke's pouch (Figure 1Q, **I**), that give rise to the anterior

pituitary gland, vestibulocochlear ganglion (Figure 1R, **I**), trigeminal ganglia and dorsal root ganglia (Figure 1S-T, **I**). MANF positive cells were also observed in the developing peripheral tissues such as heart (Figure 1U, **I**), lung (Figure 1V, **I**), liver (Figure 1X, **I**), intestine (Figure 1W, **I**), and pancreas (Figure 1Y, **I,** Figure 1**, III**).

Taken together, we observed the ubiquitous expression of MANF in most mouse tissues during the embryonic development, suggesting an essential role for MANF during embryogenesis.

4.2 MANF expression in the CNS and PNS

(Original publication **I**)

In the current work, we presented a comprehensive study of MANF expression in the mouse brain, using MANF antibodies validated by using *Manf*$^{-/-}$ tissue as negative controls. Previously, MANF expression within the CNS was studied in the embryonic heads (E17, E18.5) or whole embryos (E13, 17) and brains at P1, P10, and adult in the mouse species (Lindholm et al., 2008; Tseng et al., 2017). On top of that, a recent study by Wang et al. identified that MANF is expressed in the developing rat brain and it was suggested that MANF expression declines with the rat brain maturation (Wang et al., 2014).

Consistent with previous studies (Lindholm et al., 2008; Wang et al., 2014), we observed a positive MANF expression in different brain areas at P14 (Figure 3,Supplementary Figure 2, **I**), including cerebral and prefrontal cortex, olfactory bulbs with high levels of MANF in mitral cell layer and anterior olfactory nucleus (Supplementary Figure 2A-B, **I**), striatum (Supplementary Figure 2D, **I**), subventricular zone, thalamus (Supplementary Figure 2I, **I**), CA1-CA3 hippocampal pyramidal neurons as well as in dentate gyrus (Supplementary Figure 2E-G, **I**), choroid plexus (Supplementary Figure 2H, **I**), substance nigra (Supplementary Figure 2J, **I**), cerebellum (Supplementary Figure 2K, **I**). MANF expression was found in the neuronal subpopulation but not in glial cells studied by IHC (Supplementary Figure 2Q-S, T-U, **I**). MANF positive cells were colocalized with TH-positive dopamine neurons in the substantia nigra as also shown by Lindholm et al. (Supplementary Figure 2W-Y, **I**) (Lindholm et al., 2008). MANF expression was also found in the calbindin-positive cell bodies of cerebellar Purkinje cells and their dendrites in agreement with an earlier report (Supplementary Figure 2Z-BB, **I**) (Yang et al., 2014a).

Notably, we identified that MANF is highly expressed in the cells of various CNS centers containing glucose sensing cells, including mesocortical/mesolimbic dopamine system, different areas of hypothalamus and brainstem structures of the mouse brain at P14 (Figure 3, Supplementary Figure2, **I**).

The hypothalamus regulates food intake, energy balance, and endocrine systems through the pituitary gland (Kelberman et al., 2009; Roh et al., 2016). Within the hypothalamus, abundant expression of MANF was detected in the medial preoptic area (Figure 3C, **I**), paraventricular hypothalamic nuclei (Figure 3D, **I**), supraoptic nuclei (Figure 3E, **I**), dorsomedial nucleus (Figure 3F, **I**), arcuate hypothalamic nucleui and median eminence (Figure 3G, **I**) and ventromedial hypothalamic nuclei (Figure 2H, **I**). Furthermore, we identified that MANF positive cells are co-expressed with oxytocin (Figure 3S-U, **I**) and

vasopressin (Figure 3V-X, **I**) positive cells in paraventricular hypothalamic nuclei and supraoptic nuclei parts of the hypothalamus.

Inside the brain stem structures, which are also known for the regulation of food intake, we identified high levels of MANF in the locus coeruleus nucleus (Figure 3M, **I**), dorsal nucleus of the vagus nerve (Figure 3R, **I**), rostral ventrolateral medulla (Figure 3O, **I**) and low levels in parabrachial nucleus (Figure 3N, **I**), area postrema (Figure 3P, **I**), sensory nucleus of the solitary tract (Figure 3Q, **I**). Additionally, MANF expression is detected in circumventricular organs, such as subcommissural organ (Supplementary Figure 2L, **I**), AP. We identified that MANF is co-expressed with acetylcholinesterase positive motor neurons of the dorsal nucleus of the vagus nerve part of the mouse brain (Figure 2Y-AA, **I**), which regulates secretion and motility within the gastrointestinal tract, and secretion within the pancreas (Mussa and Verberne, 2013).

Additionally, we found MANF expression within the mesolimbic and mesocortical dopaminergic pathways that regulate hedonic feeding. Moderate expression of MANF was observed in the medial prefrontal cortex (Figure 3I, **I**), paraventricular nucleus of the thalamus (Figure 3K, **I**), nucleus accumbens (Figure 3J, **I**) and ventral tegmental area (Figure 2L, **I**). We identified that MANF is co-expressed with all TH-positive dopamine neurons in the ventral tegmental area (Figure 3BB-DD, **I**).

Descending neuronal projections from hypothalamus via brainstem structures to the intermediolateral column of the spinal cord connect with the autonomic nervous system are known to regulating heart rate, respiration, brown adipose tissue (BAT)/thermogenesis, and white adipose tissue (WAT)/energy state. We identified MANF expression in the adult mouse spinal cord (Figure 4A-E, **I**). MANF positive cells were detected within the grey matter in the glial cells and within the white matter in the myelinated axons. MANF expression was observed in neuronal subpopulations of the dorsal and ventral horns of the mouse spinal cord. Especially strong expression of MANF was detected in the motor neurons and their dendrites immuno-labeled with acetylcholinesterase and neuroendocrine PGP9.5 markers (Figure 4B, 4F-H, 4I-K, **I**).

Additionally, we observed a high intensity of MANF staining within the peripheral nervous system. Strong MANF expression was detected in the neuronal bodies of the dorsal root ganglion (Figure 4L-N, 4O-Q, 4R-T, **I**), which is known for its regulation of thermogenesis in BAT (Ryu et al., 2015). MANF expression was also high in the neurons of celiac ganglia (Figure 4U-W, 4X-Z, 4AA-CC, **I**), a component of the autonomic nervous system that regulates functions the digestive tract, liver, and pancreas.

Initially, MANF was identified as a potent factor for dopamine neurons and previous studies pointed towards MANF protective and restorative effects in different neuronal populations *in vitro* and *in vivo* mainly focusing on neurodegenerative diseases. Herein, we detected MANF expression in the CNS centers that are responsible for the homeostatic and hedonic regulation, suggesting widespread roles for MANF in regulating energy homeostasis by controlling food intake and glucose metabolism in peripheral tissues, as well as metabolic function, thermogenesis, heart rate, respiration and digestion. Lately, MANF was shown to influence food intake and body weight by regulating insulin signaling in the mouse hypothalamus (Yang et al., 2017) (chapter 1.4.4). Overexpression or knockdown of

hypothalamic MANF resulted in hyperphagia or hypophagia, respectively. Still, the role of the hypothalamic MANF expression in the control of energy balance remains unclear as randomly fed neuron-specific *NestinCre::Manf*$^{fl/fl}$ mice do not show any signs of impaired insulin signaling (chapter 5.3.3), (Lindahl et al., 2014)(Pakarinen et al., manuscript). Moreover, the role of MANF in the mesocortical/mesolimbic dopamine system, brainstem structures, spinal cord, and autonomic nervous system requires further investigation.

4.3 MANF expression in endocrine tissues

(Original publication **I**)

Results of previous work revealed that MANF is expressed at high level in secretory tissues (Lindholm et al., 2008). Therefore, we carefully investigated MANF expression in the mouse endocrine system including the hypothalamus, pituitary gland, pineal body, thyroid, and parathyroid glands, adrenal glands, pancreas and the reproductive organs (ovaries and testes). The endocrine system controls growth and developmental processes, homeostasis, metabolism, reproduction, and response to stress and injury stimuli. High level of MANF expression was observed in mouse pituitary gland, especially strong levels were detected in adenohypophysis and in the intermediate lobe (Figure 5A-C, **I**). MANF positive cells were also found within the neurohypophysis (Figure 5D, **I**). The pituitary gland is a central gland in the endocrine system because it controls the functions of other hormonal glands and regulates growth, reproduction, endocrine functioning and functions to convey signals from the hypothalamus to various organs (Kelberman et al., 2009). The adenohypophysis consists of five types of cells that secrete different hormones: somatotropes secrete growth hormone (GH), lactotropes prolactin (PRL), gonadotropes follicle stimulating hormone (FSH) and luteinizing hormone (LH), corticotropes adrenocorticotropic hormone (ACTH), and thyrotropes thyroid-stimulating hormone (TSH) (Ooi et al., 2004). We identified the co-localization of MANF expressing cells with GH and PRL secreting cells in the anterior pituitary gland at 6 weeks of age (Figure 6 G-I, 6J-L, **I**). The role of MANF in mouse adenohypophyses will be discussed in chapter 5.2 and 5.4. The mouse intermediate lobe of the pituitary gland is composed of melanotropes, which produce and secrete an α-melanocyte-stimulating hormone (α-MSH), a central regulator of energy homeostasis, and β-endorphin. Neurohypophysis mainly consists of neuronal projections from the hypothalamus and is responsible for the secretion of vasopressin and oxytocin to the bloodstream.

We also found strong MANF immunoreactivity in both thyroid and parathyroid glands (Figure 5E-G, **I**). MANF was highly expressed by the thyroid follicular cells responsible for the synthesis of thyroxine (T4), triiodothyronine (T3) as well as in calcitonin positive C-cells (Figure 5H-J, **I**) in the thyroid gland. MANF was also high in parathyroid (PTH) cells. In the adrenal gland, MANF was expressed both in the cortex and at lower levels in the medulla (Figure 5K, **I**). We demonstrated that MANF was co-expressed with TH positive chromaffin cells in the medulla (Figure 5L-N, **I**) that are responsible for the synthesis and release of catecholamines, adrenaline, and noradrenaline, in response to stress.

We observed strong immunoreactivity of MANF in the mouse pancreas. The detailed analysis of MANF expression in the pancreas will be discussed in chapter 4.2.1.

Next, we analyzed MANF expression in the mouse reproductive system. We observed MANF expression in the female mouse ovaries and ampullas (Figure 5O-Q, **I**). Notably, exceptionally high expression of MANF was observed in granulosa cells (Figure 5P, **I**), which surround the oocyte in the ovary. Additionally, we detected MANF expression in male gonads, specifically in the seminiferous tubules as also previously reported by Lindholm et al. (Figure 5R, **I**) (Lindholm et al., 2008). Hence, MANF expression in mouse tissues of the reproductive system might be needed for proper fertility.

To conclude, we detected extremely high level of MANF expression within the secretory cells of the mouse endocrine system, suggesting that MANF has important functions within cells with high production and secretion of various proteins and hormones. In addition, , the pattern of high MANF expression within the endocrine axes such as hypothalamic–pituitary–thyroid, hypothalamic–pituitary–adrenal/interrenal and hypothalamic–pituitary–gonadal axes suggest that MANF plays a crucial role in hormonal regulation thereby modulating energy metabolism, growth, development, stress responses and reproductive processes. Consequently, our studies on MANF-deficient mice revealed important roles for MANF in the mouse pancreas and pituitary gland (Chapter 5.2). Previous studies revealed that MANF deficiency in *C. elegans* led to fewer descendants, providing evidence that MANF is required for proper function of reproductive system in *C. elegans* (chapter 1.4.3) (Richman et al., 2018). Interestingly, increased expression of MANF was observed in the mouse oocytes after *in vitro* maturation (Wang et al., 2011), possibly in response to stress due to the procedure. However, more research is required to clarify the functions of MANF in other type of endocrine tissues.

4.3.1 MANF expression in the mouse pancreas

(Original publication **I**, **II**, **III**)

Pancreas consists of exocrine tissues and dispersed endocrine islets of Langerhans. The exocrine part is composed of the ductal and acinar cells, which synthesize and secrete digestive enzymes such as carboxypeptidase, amylase, and lipase into the pancreatic duct that connects to the duodenum. Islets of Langerhans consist of several endocrine cell types each secreting different hormones: alpha-cells, beta-cells, delta-cells, and pancreatic polypeptide (PP)-cells, which are responsible for the production and secretion of glucagon, insulin, somatostatin, and pancreatic polypeptide respectively. The majority of the cells within the endocrine islets are the beta-cells, which occupy 70–80% (Brissova et al., 2005) of the islet and which by secreting insulin into the blood stream regulate the blood glucose levels.

High levels of MANF was observed in the developing pancreas during differentiation processes in E13.5 mouse embryo by IHC and confirmed by positive LacZ staining and IHC in E12.5 (Figure 1Y, **I**, Figure 1A, Supplementary Figure 1.1A, Supplementary Figure 2.2K, 2.2O, **III**). We also observed MANF expression in the pancreatic islets and exocrine acinar cells at E18.5, P1, P3 and P14 addressed by IHC (Supplementary Figure 2.2L, 2.2P, 2.2M, 2.2Q, 2.3A, 2.3D, **III**). Our studies revealed that in comparison with other mouse tissues, the highest expression levels of MANF protein and mRNA were found in the endocrine islets and pancreatic exocrine acinar cells of the adult mouse pancreas analyzed by western blotting

(Supplementary Figure 4A-B, **II**, Figure1 B, Supplementary Figure 1.1C, **III**). MANF mRNA and protein in mouse pancreatic islets and exocrine tissue were relatively the same (Figure 1E, 1F, **III**). Similarly to mouse pancreas, we also identified immunoreactivity of MANF in the endocrine islets and exocrine acinar cells of the human pancreas (Supplementary Figure 4G-I, **II**). Importantly, double-IHC on pancreatic sections from adult mice, revealed that MANF was mainly localized in the beta-cells (Figure 1G-I, **III**), less in delta-cells (Figure 1K-M, **III**), but was not detected in alpha- or PP-cells (Figure 1O-Q, 1S-U, **III**). In support to our data, MANF expression was observed in beta-cells and also delta-cells but not in alpha-cells in human pancreas (Hakonen et al., 2018), although human *MANF* mRNA was observed in both beta-cells and alpha-cells in similar levels (Segerstolpe et al., 2016). This result suggest that translation of *MANF* mRNA does not occur in alpha-cells.

PDX1 is a key regulator for pancreatic development and beta-cell maturation (Fujimoto and Polonsky, 2009). During pancreas development, PDX1 marks the progenitors of all exocrine and endocrine cell types at E8.5-E9.5 in mouse embryo (Ohlsson et al., 1993). In adult pancreas, PDX1 expression becomes restricted to the beta-cells (Ashizawa et al., 2004). GLUT2 is expressed at the cell membrane in the mouse beta-cells and required for the insulin secretion stimulated by glucose (Thorens, 2015). In the mouse islets MANF positive cells co-expressed with pancreatic and duodenal homeobox 1 (PDX1) in the nucleus (Supplementary Figure 1.1H-J, **III**) as well as GLUT2 (Supplementary Figure 1.1E-G, **III**), proving that indeed MANF is expressed in beta-cells.

The *in vivo* role of MANF in the mouse pancreas will be discussed in chapter 5.2.

4.3.2 Cellular localization of MANF in the mouse primary beta-cells and MIN6 cell line

(Original publication **III** and unpublished results)

Previous studies revealed MANF localization in the ER of the neurons and immortalized cell lines (Glembotski et al., 2012; Henderson et al., 2013; Matlik et al., 2015).

In order to investigate the subcellular localization of MANF in the beta-cells, we performed confocal analysis of the mouse primary islets cells stained with insulin, PDI (protein disulfide isomerase, a marker for ER), GM130 (a marker for cis-Golgi), GRP78, and MANF. We observed robust co-localization of MANF with insulin in the ER (Figure 1W, 1Y, Supplementary Figure 1.1 K-S, 1.1 M-U, **III**), a limited co-localization of MANF with GRP78 (Figure 1Z, Supplementary Figure 1.1 N-V, **III**) and complete separation of MANF from Golgi (Figure 1X, Supplementary Figure 1.1 L-T, **III**) in unstressed cells based on the Mander's and Pearson correlation coefficients (Table 2, **III**). Consistent with the results on primary beta-cells, we observed co-expression of MANF with PDI as a marker for ER and GRP78, partial co-expression with insulin and segregation with GM130 used as a marker for Golgi in mouse insulinoma MIN6 cell line (Figure 4). Furthermore, our collaborative studies confirmed MANF localization in the ER of the human beta-cell line EndoC-βH1 (Hakonen et al., 2018). Taken together, the results confirm that MANF is localized in the ER of both mouse and human beta-cells. However, MANF is secreted from human beta-cell line EndoC-βH1upon cytokine treatment (Hakonen et al., 2018), suggesting that secretion of MANF is destined via the Golgi apparatus transport.

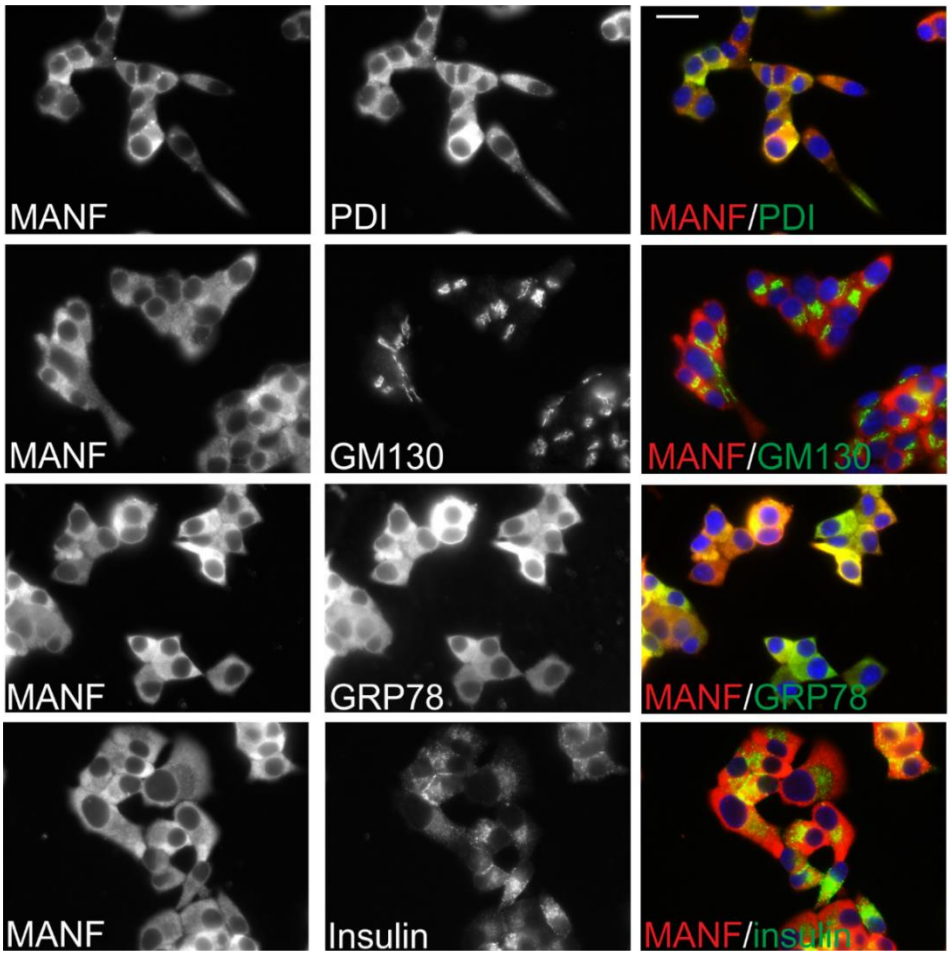

Figure 4. Representative microscopy images of MIN6 cell line labeled with MANF (red) and co-stained with PDI (ER marker, green), GM130 (Golgi marker, green), GRP78 (green) or insulin (green) antibodies. Scale bar, 20 µm.

4.4 MANF expression in other tissues

(Original publication **I**)

Within the organs with exocrine functions, strong MANF expression was detected in all parts of mouse salivary gland including sublingual gland, parotid gland, and submandibular gland (Supplementary Figure 4A-C , **I**). Notably, MANF was detected in the mucous acini and serous acini of the submandibular gland and secretory acini of the parotid gland. Variable expression of MANF was detected in mouse lungs (Supplementary Figure 4D, **I**), which consists of epithelial, endothelial and hematopoietic cells. High expression of MANF was observed in tracheal and esophagus epithelium cells (Supplementary Figure 4E, 4F, **I**). In mouse skin, we observed MANF immunoreactivityin the epithelium, sebaceous glands and the cells of the dermis (Supplementary Figure 4G, **I**). Taken together, strong MANF expression were observed in the organs with exocrine functions and epithelial cells of several tissues, suggesting that MANF regulates secretory functions and thus might promote epithelial barrier function of these tissues.

In the liver, low MANF expression was detected by IHC in hepatocytes (Supplementary Figure 7J, **I**). In the kidney, high immunoreactivityof MANF was observed in the cells of the

juxtaglomerular apparatus compared with low levels in the tubules (Supplementary Figure 7G-I, **I**). These results suggest possible roles for MANF in renin–angiotensin–aldosterone hormonal system that regulates blood pressure and fluid and electrolyte balance in the body.

We also observed MANF expression throughout the gastrointestinal tract (GI). Specifically, MANF positive immunoreactivity was identified in epithelial cells of the villi in the duodenum (Supplementary Figure 4H, **I**). The high intensity of MANF staining was observed in certain cells including enterocytes, mucus secreting goblet cells, and crypt Paneth cells (Supplementary Figure 4I-K, 4L-N, 4O-Q, **I**). So, MANF might have a role in intestinal epithelial barrier function and antigen uptake.

Previous studies identified MANF protective role for cardiomyocytes simulated ischemia and heart ischemia (chapter 1.3.4) (Tadimalla et al., 2008; Glembotski et al., 2012). Moreover, it demonstrated increased MANF expression in the mouse heart after ischemia (Tadimalla et al., 2008). We found only a low number of MANF immune-reactive cells in cardiac muscle and atrium tissue of the mouse heart (Supplementary Figure 5A-C, **I**), and in muscle (Supplementary Figure 5E, **I**), suggesting that MANF might not have a direct function in these tissues, but acting in a stress-induced conditions.

MANF positive cells were also detected in epicardial adipose tissue (Supplementary Figure 5D, **I**). In addition, we detected MANF immune-reactive cells in adipocytes of WAT (Supplementary Figure 5F, **I**) and BAT (Supplementary Figure 5G, **I**). These results together with the previous finding (chapter 4.2, MANF expression in CNS and PNS) further supports that MANF might be important for the highly metabolic cells that regulate thermogenesis and modulates energy state in mice.

We also detected MANF expression within the mouse lymphatic system including spleen, thymus, lymph node, and intestinal lymphatic tissue - Peyer's patches, which are known for its regulation of the adaptive and innate immune system. We observed MANF expression mainly within the red pulp and marginal-zone in the spleen in agreement with an earlier report (Supplementary Figure 6A-B, **I**) (Liu et al., 2015a). Within the thymus, MANF expression was mainly detected in the medulla (Supplementary Figure 6C-D, **I**). We also detected MANF positive cells in mouse lymph node (Supplementary Figure 6E-F, **I**) and small lymphatic tissue of the intestine Peyer's patch (Supplementary Figure 6G-H, **I**). Expression of MANF within the lymphatic system implicates a possible function of MANF in the immune system. Certainly, previous studies documented anti-inflammatory properties of MANF (chapter 1.3.3). On the other hand, the immune cells are highly secretory cells that have a high demand for folding of the proteins in the ER (Grootjans et al., 2016). Hence, MANF expression in lymphatic tissues could be required for maintaining the ER homeostasis in the immune cells. However, more research is required to investigate the role of MANF within the lymphatic system.

Table 3. Summary table of MANF expression in adult mouse tissues by different techniques.

Mouse tissue	Relative *Manf* mRNA level/β-actin	MANF protein levels, ng/mg total protein by ELISA	IHC MANF
Pituitary gland	++++	++++	+++++
Thyroid gland	++	++	+++++
Adrenal gland	++	++	++++
Pancreatic islets	+++++	not analyzed	+++++
Pancreatic exocrine tissue	+++++	not analyzed	+++++
Testis	++++	+++++	+++++
Ovary	+++	++	+++
Brain	+	+++	+++
Thymus	+	+	++
Lung	++	not analyzed	+
Heart	++	+	+
Liver	+++	++++	+++
Salivary gland	+++	++++	+++++
Kidney	+	++	+
Spleen	+	++	+
Duodenum	+++	not analyzed	+++
Jejunum	+++	not analyzed	not analyzed
Ileum	++	not analyzed	not analyzed
Colon	++	not analyzed	not analyzed
Muscle	++	+	+
Brown adipose tissue (BAT)	not analyzed	+++	++
White adipose tissue (WAT)	not analyzed	+	+

++++ - very strong expression, +++ - strong expression, ++ - moderate expression, + - low expression

4.5 CDNF expression in mouse tissues

(Original publication **I**)

MANF and CDNF are homologous proteins with seemingly distinct functions (Lindahl et al., 2017). Therefore we wanted to know whether they are differently expressed in mouse tissues by extensive comparative analysis of mRNA and protein expression using qPCR and ELISA.

So far, we were not able to identify an anti-CDNF antibody that would specifically recognize endogenous CDNF in mouse tissues by IHC. However, in this study, we analyzed CDNF expression in mouse tissues by qPCR and in-house developed CDNF ELISA. Consistent with previous studies, we identified that the levels of CDNF protein in mouse tissues are significantly lower compared to the levels of MANF protein (Table 4). High *Cdnf* mRNA levels were observed in mouse testis, pituitary gland and exocrine tissue of the pancreas, heart, and muscle compared to relatively low levels in the rest of analyzed tissues

(Figure 2C, **I**). CDNF protein is highly expressed in mouse heart, muscle, testis, and BAT (Figure 2D, **I**). Moderate CDNF protein levels were observed in the pituitary gland, adrenal gland, kidney, ovary, brain, thymus and WAT compared with low levels in pancreas, liver, salivary gland, and spleen (Figure 2D, **I**).

These differences in expression patterns indicate to partly distinct roles for MANF and CDNF *in vivo*. In support of this hypothesis, opposite to phenotypes of *Manf*$^{-/-}$ mice, we did not observe apparent defects in growth, diabetes or the lifespan of *Cdnf*$^{-/-}$ conventional knockout mice (Lindahl et al., unpublished data).

Table 4. Comparison of MANF and CDNF protein expression in mouse tissues using ELISA.

Mouse tissue	MANF protein levels, ng/mg total protein	CDNF protein levels, ng/mg total protein
Pituitary gland	338.6±3.91	1.26±0.19
Thyroid gland	139.0±3.37	30.64±1.66
Adrenal gland	134.2±3.75	2.55±0.47
Pancreas	894.1±9.41	0.09±0.03
Testis	1169.5±9.16 /	10.77±0.84
Ovary	165.1±3.4	0.77±0.18
Brain	247.6±2.79	0.76±0.19
Thymus	123.4±2.03	0.89±0.23
Lung	not analyzed	not analyzed
Heart	38.5±0.99	18.84±0.84
Liver	375.7±4.65	0.12±0.09
Salivary gland	320.1±5.97	0.28±0.14
Kidney	176.8±1.38	1.42±0.21
Spleen	187.6±0.8	0.3±0.09
Duodenum	not analyzed	not analyzed
Jejunum	not analyzed	not analyzed
Ileum	not analyzed	not analyzed
Colon	not analyzed	not analyzed
Muscle	81.8±1.67	19.22±0.86
Brown adipose tissue (BAT)	198.7±2.63	11.91±0.47
White adipose tissue (WAT)	66.6±2.63	1.54±0.3

4.6 Upregulation of MANF and CDNF expression in ER stress conditions *in vivo*

4.6.1 MANF expression is upregulated in the beta-cells of diabetic mice

(Original publication **III**)

Previous studies identified increased levels of MANF mRNA and protein in the beta-cells of Ins2^{Akita} mice, where misfolded proinsulin accumulates in the ER thereby triggering initiation

of ER stress and UPR resulting in the apoptosis of beta-cells (Oyadomari et al., 2002a; Oyadomari et al., 2002b; Mizobuchi et al., 2007; Balboa et al., 2018; Riahi et al., 2018). Islets isolated from pre-diabetic NOD mice display enhanced *Manf* mRNA and activation of the UPR that was found to precede the beta-cell death (Tersey et al., 2012; Morita et al., 2017). In agreement with this data, we identified increased MANF expression in the beta-cell close to insulitis area in pre-diabetic 12-week-old pancreas from NOD mice (Supplementary fig 1.2D-F, 1.2 J-L, **III**). MANF expression was not detected in the insulitis area in the NOD islets (Supplementary fig 1.2D-F, 1.2 J-L, **III**). Other studies documented increased expression of *Manf* mRNA in the beta-cells of db/db mice at 12 weeks of age by RNA sequencing (Neelankal John et al., 2018). Consistently, in our studies, we reported that MANF expression is upregulated in beta-cell of db/db mice at 8 weeks of age by IHC (Supplementary Figure 1.2Q-S, 1.2 W-Y, **III**).

Recent evidence indicates MANF protective effects against ER stress in beta-cells. Diminished *Glis3* expression in beta-cells of immune-independent NOD mice led to decrease expression of MANF, where chronic ER stress and beta-cell apoptosis was caused by transgenic overexpression of hen lysozyme, suggesting that transcription factor Glis3 could regulate the expression of the *Manf* gene (Dooley et al., 2016). Reduced or lack of Glis3 expression leads to neonatal diabetes in mouse and human (Senee et al., 2006; Watanabe et al., 2009). In contrast, *Manf* expression was upregulated in normal C57BL/6 mouse beta-cells with ER stress caused by the hen lysozyme transgene expression (Dooley et al., 2016). However, upon HFD, *Glis3* and *Manf* mRNA and protein expression were reduced, resulting in beta-cell apoptosis and diabetes (Dooley et al., 2016). Consequently, levels of MANF expression in beta-cells correlated with beta-cell survival in normal, diabetes-susceptible and stressed conditions. However, the exact relevance for MANF levels in diabetic beta-cells requires further exploration.

5. Genetic ablation of MANF in mice

To understand the biological role of MANF, a detailed analysis of MANF conventional knockout (*Manf*$^{-/-}$) mice and MANF conditional knockout mice was performed.

5.1 Generation of *Manf*$^{-/-}$ mice

(Original publication **II** and **III**)

The MANF knockout mice (*Manf*$^{-/-}$) were developed from a targeted embryonic stem (ES) cell clone MANF_D06 (EPD0162_3_D06; C57Bl/6N-Manf $^{tm1a(KOMP)Wtsi}$) that contained an efficient splice acceptor-site followed by a beta-galactosidase cassette inserted in the intron between exon 2 and exon 3 of the *Manf* gene, thus resulting in a constitutive null mutation through the splicing of exon 2 to the reporter cassette (Figure 1 A, **II**), confirmed by RT-PCR and Western blotting analysis (Supplementary Figure 1A, 1 B, **II**). The targeted *Manf* mutant allele in the *Manf*$^{-/-}$ mice contains Frt-sites that allowed removal of the strong splice-acceptor and beta-galactosidase cassette by the crossing of *Manf*$^{+/-}$ to globally expressing CagFlp-recombinase transgenic mice (Supplementary Figure 2A, **II**, Supplementary Figure 2.1 A,

III). The resulting mice (*Manf*$^{fl/fl}$) are healthy and express *Manf* mRNA in tissues equally to *Manf*$^{+/+}$ mice (Supplementary Figure 2B, **II**). In these mice, the Lox P sites (floxed) on both sides of exon 3 in the *Manf* locus enable conditional inactivation of the *Manf* gene by the crossing of mice to specific Cre-recombinase expressing mice.

5.2 Absence of MANF causes diabetic phenotype and growth defect in conventional *Manf*$^{-/-}$ mice

(Original publication **I**and **II**)

We found that MANF deficiency leads to a severe growth retardation and poor survival in both male and female mice in mixed Hsd:ICR(CD-1);C57BL/6 background (Figure 1 B, **II**). *Manf*$^{-/-}$ mice are significantly smaller already at E18.5 and continue to show weak growth (Figure 1 B, **II**). In addition, about 25% of the MANF-deficient mice die soon after birth whereas the rest of *Manf*$^{-/-}$ mice start to show signs of sickness between 8-11 weeks of age and most die before 12 weeks of age.

The metabolic phenotype of 6-week-old MANF-deficient mice compared to WT mice was assessed for 60 h (2 days and 3 nights) in a Comprehensive Laboratory Animal Monitoring System (CLAMS). We observed that *Manf*$^{-/-}$ mice consume more water (Supplementary Figure 1G, **II**), although food intake appears to be similar between the genotypes (Supplementary Figure 1H, **II**). Consumption of O_2 and CO_2 was significantly decreased in MANF-deficient mice compared to WT mice both during day and night (Supplementary Figure 1I, 1J, **II**). The respiratory exchange ratio was decreased in MANF-deficient animals during the night (Supplementary Figure 1K, **II**), indicating that mainly fat is utilized as an energy substrate. In addition, we observed no changes in locomotor activity in open field arena between the genotypes (Supplementary Figure 1L, **II**).

The metabolic phenotypes in *Manf*$^{-/-}$ indicated hyperglycemia and consequently blood glucose measurements showed that *Manf*$^{-/-}$ mice suffer from severe insulin-deficient diabetes. Signs of hyperglycemia and insulin deficiency were first noticed in random fed *Manf*$^{-/-}$ mice at P28 (Figure 5A, 5B) (Figure 1C, 1E, **II**), and the symptoms progressed with age. To confirm that the diabetic phenotype was not caused by the insertion of the beta-galactosidase cassette in the mouse genome, we first removed the beta-galactosidase cassette by crossing *Manf*$^{+/-}$ mice to CagFlp- expressing mice to generate *Manf*$^{fl/+}$ mice. Then we ubiquitously removed *Manf* from all cells by crossing of *Manf*$^{fl/fl}$ mice with PgkCre transgenic mice. Consequently, we observed a similar diabetic phenotype with a severe growth retardation in *PGKCre::Manf*$^{fl/fl}$ mice, confirming that the beta-galactosidase cassette did not cause the evident phenotypes in the *Manf*$^{-/-}$ mice. (Supplementary Figure 2C-E, **II**). On the contrary, recent studies revealed that global deletion of MANF in mice on C57BL/6 background led to perinatal lethality due to defects in breathing caused by reduced lung alveolar volume and abnormalities in lung development (Neves et al., 2016; Bell et al., 2019). In agreement, decreased survival of *Manf*$^{-/-}$ mice was detected in litters with increased rounds of backcrossing of *Manf*$^{+/-}$ mice in the ICR outbred strain to the inbred C57BL6 strain.

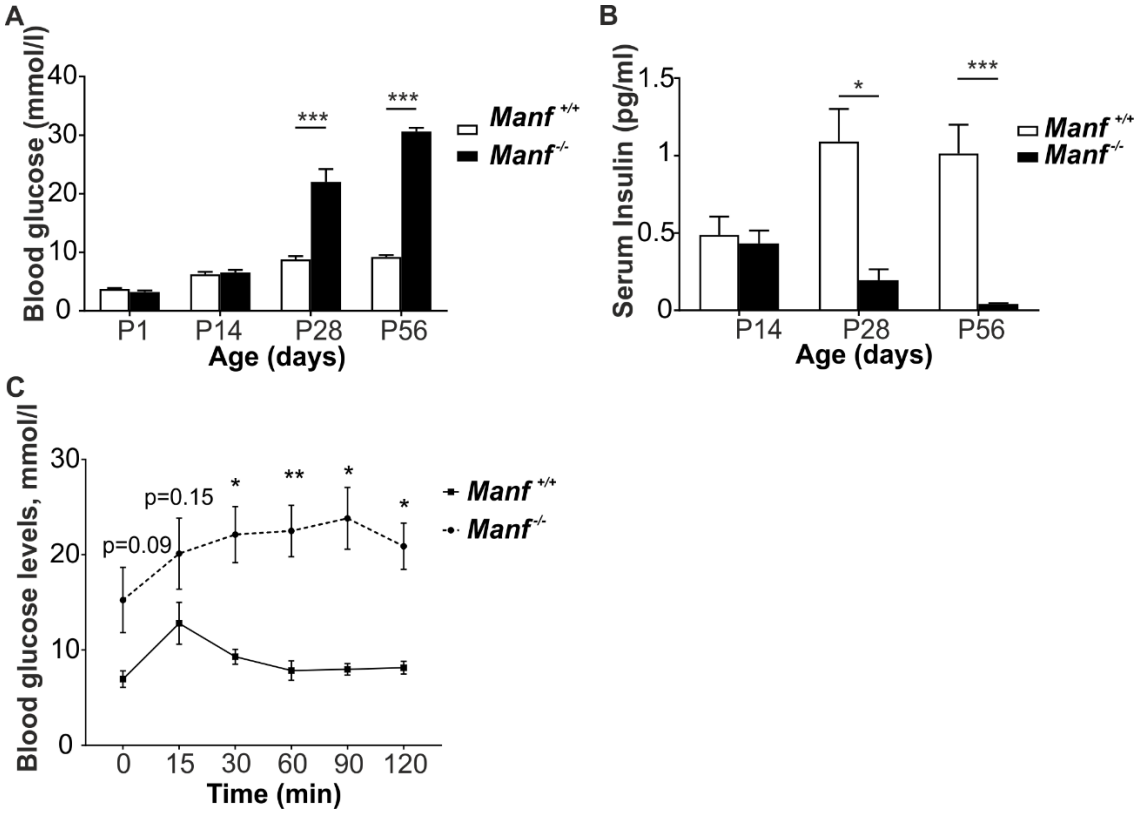

Figure 5. MANF-deficient mice develop diabetes. **(A)** Blood glucose levels measured in *ad libitum*-fed *Manf*$^{-/-}$ mice, and their controls, *n* = 11-12 animals per group, both genders. **(B)** Serum insulin levels measured in *Manf*$^{-/-}$ mice and their controls from *ad libitum*-fed mice, *n* = 11-12 mice per group, both genders. **(C)** Blood glucose levels measured after intraperitoneal glucose (2 g/kg) injection in 8 weeks old mice, *n* = 4 mice per group.

Next, we performed physiological tests including glucose tolerance test (GTT) and insulin tolerance test (ITT). GTT measures the function of the beta-cells and insulin sensitivity, while ITT is used to addresses the insulin sensitivity of the whole body (Bowe et al., 2014). Our results revealed impaired glucose clearance and intact insulin sensitivity in the knockout animals at P56 (Figure 1F, **II**) (Figure 5C). Signs of impaired beta-cell function was detected already in 2-week-old *Manf*$^{-/-}$ mice as glucose challenge test showed decreased blood glucose clearance (Figure 1D, **II**) and barely detectable serum insulin levels in 8-week-old *Manf*$^{-/-}$ measured by ELISA (Figure 1G, **II**). To investigate, whether the function of beta cells from *Manf*$^{-/-}$ mice was affected, we analyzed insulin secretion on isolated islets after glucose stimulation *in vitro* (Figure 1H, 1I, **II**). The islets from diabetic MANF-deficient mice secreted significantly less insulin than *Manf*$^{+/+}$ control islets at P35 under glucose stimulation for 1 hour. However, the ability of *Manf*$^{-/-}$ islets to secrete insulin in relation to total islet insulin content was not affected meaning that the capacity of *Manf*$^{-/-}$ beta-cells to secrete insulin was not reduced. In addition, the mRNA levels of glucokinase enzyme which phosphorylates glucose to glucose-6-phosphate important for ATP production and insulin release, was not reduced in islets isolated from *Manf*$^{-/-}$ mice (Figure 3, **II**). Therefore, we set out to study whether hyperglycemia was caused by loss of pancreatic beta-cells in *Manf*$^{-/-}$ mice.

Histological analysis of the *Manf*$^{-/-}$ pancreas revealed that the beta-cell mass in pancreases from embryonic E18.5 *Manf*$^{-/-}$ mice was similar to beta-cell mass in wt mice. However, at P1 the beta-cell mass had dropped by 50% compared to the control mice (Figure 2A-F, 2G, **II**). Notably, the glucagon-positive alpha-cell mass was not reduced in the *Manf*$^{-/-}$ pancreases at any developmental stage quantified (Supplementary Figure 3D-I, 3G, **II**). However, the alpha-cells were no longer located in the border of islets but dispersed inside the islets, reflecting the progressive loss of beta-cells and disturbed islet architecture.

The existing beta-cell mass results from a balance between the processes of beta-cell replication and beta-cell apoptosis. The beta-cells proliferate at the highest levels in mouse neonates, while this process declines with age (Teta et al., 2005).

To reveal the mechanisms behind the reduction in beta-cell mass, we first set out to study, in pancreatic sections from mice, the rate of beta-cell proliferation (by quantification of the number of Ki67-positive beta-cells) and apoptosis (by TUNEL).The number of proliferating beta-cells was significantly reduced in the *Manf*$^{-/-}$ pancreas at P1 and P14 mice compared to *Manf*$^{+/+}$ mice, whereas no reduction could be detected in embryonic E16.5 and E18.5 *Manf*$^{-/-}$ islets (Figure 2H, **II**), indicating that ablation MANF has no effect on beta-cell differentiation. However, even though MANF is highly expressed in pancreatic exocrine acinar cells, the proliferation rate of acinar cells was not affected in *Manf*$^{-/-}$ mice (Supplementary Figure 3B, **II**), suggesting that MANF is critically needed for proper beta-cell proliferation. To verify if beta-cell death contributes to reduced beta-cell mass, TUNEL staining followed by insulin staining on *Manf*$^{-/-}$ pancreases showed that the number of dying islet beta-cells was significantly increased in *Manf*$^{-/-}$ pancreas at P14 and P56 (Figure 2J, Supplementary Figure 3C, **II**).

To conclude, our results suggest that the progressive hyperglycemia in *Manf*$^{-/-}$ mice is triggered by postnatal loss of beta-cell mass due to decreased beta-cell proliferation and enhanced beta-cell apoptosis.

We showed that global MANF-deficiency in mice resulted in a severe growth defect and a significant (16%) reduction in body length measured at 8 weeks of age (Supplementary Figure 1D-F, **II**). A recent study identified that cartilage-specific ablation of MANF in mice led only to a maximum of 5% reduction in skeletal bone and body length (Bell et al., 2019). Thus, the growth defect found in our *Manf*$^{-/-}$ mice in the ICR background indicated that ablation of MANF from other cell types rather than only cartilage resulted in the dwarfism in *Manf*$^{-/-}$ mice.

In humans growth retardation or dwarfism is often caused by growth hormone (GH) deficiency (Raben, 1958). As GH is produced in the endocrine anterior pituitary and MANF was shown to be highly expressed by the adenohypophysis, we studied in more detail the *Manf*$^{-/-}$ mutant pituitary glands (Original publication **I**). *Manf*$^{-/-}$ pituitary glands revealed alteration of its cell composition compared to *Manf*$^{+/+}$ as analyzed by hematoxylin-eosin staining. Notably, adenohypophysis of *Manf*$^{-/-}$ pituitary was affected by the reduced size and by decreased staining and ratio of granule-filled acidophilic cells (somatotropes and lactotropes) compared with *Manf*$^{+/+}$ in 6 weeks old mice (Figure 6A-D, 6E, **I**).

Consequently, the number of GH-positive and PRL-positive cells were reduced in the anterior lobe of the *Manf*$^{-/-}$ pituitary gland (Figure 6M-N, 6O-P, **I**). In addition the number of

proliferating pituitary cells assessed by Ki67 IHC were reduced (Figure 6S, Supplementary Figure 7C-D, **I**). There were no detectable changes in the apoptosis rates of the *Manf*$^{-/-}$ pituitary gland compared to *Manf*$^{+/+}$ controls in 6 weeks of old mice (Supplementary Figure 7E, **I**), suggesting that the reduced ratio of somatotropes and lactotropes was caused by the reduced proliferation rate.

Our further studies revealed that *Gh* mRNA and mouse *Prl* mRNA were reduced by 50% and 79%, respectively, in mutant *Manf*$^{-/-}$ pituitaries compared to controls (Figure 6T, **I**). On the contrary levels of *Pro-opiomelanocortin (Pomc)* mRNA and *Tshβ* mRNA were significantly increased (Figure 6T, **I**). Luteinizing hormone β (*Lhβ)* and follicle-stimulating hormone *(Fsh)* mRNA levels were slightly decreased in *Manf*$^{-/-}$ pituitary glands at 6 weeks old male mice, while *Fsh* mRNA levels were significantly enhanced and *Lhβ* mRNA was not changed in *Manf*$^{-/-}$ female pituitary glands (Figure 6U, 6W, **I**). Pituitary-specific positive transcription factor 1 (Pit1) is required for the development of the anterior pituitary, particularly it plays role in the differentiation and maintenance of thyrotropes, somatotropes, and lactotropes (Cohen et al., 1996). We detected reduced levels of *Pit1* mRNA expression in MANF-deficient pituitaries (Figure 6T, **I**), confirming the deficiency of both somatotropes and lactotropes in *Manf*$^{-/-}$ pituitary glands.

Hence, our results propose that MANF is important for the maintenance of GH and PRL expressing cells in mice. Thus, MANF may have a pivotal role of MANF as a regulator of the maintenance of the acidophilic cells in the mouse pituitary gland. However, additional studies are required to elucidate the role of MANF in the regulation of growth. Interestingly, similarities with the phenotype of *Manf*$^{-/-}$ mice was also observed in C*dk4*-null mice with specific endocrine phenotypes including infertility, dwarfism and diabetes (Rane et al., 1999; Tsutsui et al., 1999). As with MANF, Cdk4 was shown not to be vital during mouse development but required for the postnatal proliferation of pancreatic beta-cells, somatotrophs and lactotrophs (Rane et al., 1999; Tsutsui et al., 1999; Jirawatnotai et al., 2004). Hence, defects in the proliferation of MANF-deficient beta-cells, somatotropes and lactotropes could be associated with alteration in CDK4.

The role of MANF in humans is not yet defined. *In vitro*, endogenous MANF seems to be replaceable for the survival of primary human beta-cells (Hakonen et al., 2018). However, when treated with inflammatory cytokines, beta-cells with reduced MANF-expression were significantly more vulnerable to beta-cell death compared to beta-cells with normal MANF levels (Cunha et al., 2017). Interestingly, a clinical exome sequencing study of Middle Eastern patients with neurocognitive phenotypes revealed a young 22-year-old patient with a homozygous missense mutation in the splice donor site in exon 1 of the human MANF gene suggesting total lack of MANF or hypomorphic MANF expression in this patient (Yavarna et al., 2015). She was reported to suffer from obesity, T2D, short stature, mild intellectual disability, microcephaly, hypothyroidism, and primary hypogonadism, myopia, and autoimmune alopecia (Yavarna et al., 2015), which partly recapitulating phenotypes of the MANF knockout mice. However, to date patients with similar mutations have not yet been reported. Increased levels of MANF in blood serum was found at the manifestation of T1D in children before puberty, although no changes were detected in older children and teenagers with recent-onset T1D or in adults with prolonged T1D (Galli et al., 2016). Moreover,

circulating MANF levels were significantly enhanced in newly diagnosed insulin resistant pre-diabetic and type 2 diabetic patients (Wu et al., 2017). However, it remains to be studied if elevated MANF in serum blood of T1D and T2D patients are related to impaired beta-cell function. Our recent study revealed that MANF secretion was stimulated by cytokines from a human beta-cell line EndoC-βH1 (Hakonen et al., 2018), thus suggesting stressed beta-cells as a source of increased MANF secretion into the bloodstream in newly diagnosed T1D and T2D.

5.3. Analysis of MANF functions in conditional knockout animals

(Original publication **III**)

We specifically deleted MANF from the embryonic pancreas by crossing *Pdx-1Cre*^{TUV} mouse line (Jackson Laboratories, Stock 014647) (Hingorani et al., 2003) to *Manf*$^{fl/fl}$ mice. To evaluate the Pdx-1Cre recombinase activity, we crossed *Pdx-1Cre* mice with *Rosa26-tdTomato*$^{fl/Stop/fl}$ reporter mice (Jackson Laboratories, Stock 007914). To address the roles for MANF in postnatal/adult beta-cells *in vivo,* we ablated MANF from the beta-cells in adult mice by using tamoxifen (Tmx)-inducible MIP1-CreERT mice (Tamarina et al., 2014) crossed to *Manf*$^{fl/fl}$ mice. 8-weeks-old *MIP1-CreERT*::*Manf*$^{fl/fl}$ animals received 33 mg/kg of Tmx for 5 consecutive days and control mice were injected with corn oil. Four weeks post injections, mice were assessed for different analysis. To ensure that diabetic phenotype of the MANF knockout mice was not caused by MANF deletion from the brain, we crossed rat promoter *NestinCre* transgenic mice (a gift from Edgar Kramer (B6.Cg-Tg(Nes-cre)1Kln/J) to *Manf*$^{fl/fl}$ mice.

5.3.1 Pancreas specific ablation of MANF in mice

(Original publication **II and III**)

The roles of MANF specifically in the mouse pancreases was addressed by crossing *Manf*$^{fl/fl}$ mice with transgenic PDX1-Cre mice.

The specificity of Pdx1Cre recombinase activity was examined in the pancreas, and other tissues of *Pdx1-Cre*$^{+/-}$ mice crossed to tdTomato reporter mice (Jackson Laboratories, Stock 007914). We observed Cre-mediated tdTomato red fluorescence signal in several mouse tissues, including pancreas, duodenum, and liver at different embryonic and postnatal stages (Supplementary Figure 2.2K-DD, **III**), although Pdx1Cre-mediated recombination was reported in the developing pancreas, antral stomach and duodenum of the newborn animals and pancreatic beta-cells postnatally (https://www.jax.org/strain/014647). No ectopic Cre activity was found in MANF expressing brain cells in the cortex, hippocampus, and hypothalamus in MANF conditional animals compared to controls (Supplementary Figure 2.2A-J, **III**), suggesting that MANF expression in the brain do not cause diabetes in *Pdx-1Cre*$^{+/-}$::*Manf*$^{fl/fl}$ mice.

In accordance with phenotype of *Manf*$^{-/-}$ mice, conditional *Pdx-1Cre*$^{+/-}$::*Manf*$^{fl/fl}$ mice develop insulin-deficient diabetes (Original publications **II**, **III**). Interestingly, we observed a slightly delayed onset of diabetes in *Pdx-1Cre*$^{+/-}$::*Manf*$^{fl/fl}$ mice compared to *Manf*$^{-/-}$ mice.

Pdx-1Cre$^{+/-}$::*Manf*$^{fl/fl}$ mice start to show a slight decrease in weight starting at P56 (Supplementary Figure 2.1B, **III**), when animals become hyperglycemic (Supplementary Figure 2.1C, **III**). This implies that the weight reduction is caused by the diabetes and that ablation of MANF from other tissues rather than beta-cells results in the growth retardation seen in global *Manf*$^{-/-}$ mice.

No changes in the blood glucose levels were observed in P1 and P14 of *ad- libitum* fed *Pdx-1Cre*$^{+/-}$::*Manf*$^{fl/fl}$ mice compared to control *Manf*$^{fl/fl}$ mice (Supplementary Figure 2.1B, **III**). Acute hyperglycemia and a significant decrease in serum insulin levels were observed in P56 *Pdx-1Cre*$^{+/-}$::*Manf*$^{fl/fl}$ mice (Supplementary Figure 2.1D, **III**). To characterize a metabolic phenotype in conditional animals, we performed intraperitoneal GTT, which revealed the inability of P56 *Pdx-1Cre*$^{+/-}$::*Manf*$^{fl/fl}$ mice to clear glucose from the blood stream (Supplementary Figure 2.1E, **III**). However, we noted that glucose-stimulated insulin levels were not significantly downregulated 30 minutes after glucose injection in conditional knockout animals at P56 (Supplementary Figure 2.1F, **III**), indicating that the function of the beta-cells in *Pdx-1Cre*$^{+/-}$::*Manf*$^{fl/fl}$ mice is not completely diminished compared to their controls. As with global *Manf*$^{-/-}$ mice, ITT revealed intact insulin sensitivity in the *Pdx-1Cre*$^{+/-}$::*Manf*$^{fl/fl}$ mice at P42 (Supplementary Figure 2.1G, **III**), indicating that these mice respond to insulin by lowering of blood glucose levels.

Consistent with our previous results (**II**), we detected marked loss of islet structure and a deficit of insulin staining in the pancreases of P56 *Pdx-1Cre*$^{+/-}$::*Manf*$^{fl/fl}$ mice (Supplementary Figure 2.1I-N, **III**). In opposite to *Manf*$^{-/-}$ mice, no changes were detected in the beta-cell mass at P1 of *Pdx-1Cre*$^{+/-}$::*Manf*$^{fl/fl}$ mice, whereas a significant decrease appeared in P14 mice (Supplementary Figure 2.1U, **III**). As the beta-cell mass was not altered in MANF-deficient pancreases at E18.5 and in P1 conditional *Pdx-1*$^{Cre/+}$::*Manf*$^{fl/fl}$ mice, we suggest that MANF is not required for the differentiation of beta-cells via the processes of neogenesis or proliferation before birth. Further analysis revealed a reduced number of Ki67 positive beta-cell in P14 conditional mice, but not in P1 and P56 animals (Supplementary Figure 2.1W, **III**). Increased levels of apoptosis were detected in *Pdx-1Cre*$^{+/-}$::*Manf*$^{fl/fl}$ mice at P14 and P56 assessed by TUNEL staining (Supplementary Figure 2.1X, **III**).

Generally, our results demonstrate that embryonic ablation of MANF specifically from the pancreatic beta-cells leads to insulin-deficient diabetes caused by the postnatal reduction of the beta-cell mass due to the increased beta-cells apoptosis and decreased beta-cell proliferation. Interestingly, abnormalities in beta-cell proliferation and beta-cell apoptosis in conditional animals were detected later than in *Manf* $^{-/-}$ mice, resulting in the delayed phenotype of *Pdx-1Cre*$^{+/-}$::*Manf*$^{fl/fl}$.

The efficiency of conditional beta-cell targeting influences the experimental outcome (Magnuson and Osipovich, 2013). Consistent with other studies (Gannon et al., 2000; Herrera, 2000; Steneberg et al., 2005), we observed that *Pdx-1Cre*$^{+/-}$::*Manf*$^{fl/fl}$ mice exhibit a mosaic recombination pattern in both exocrine acinar cells and endocrine islets in adults (Figure 2F, Supplementary Figure 2.3A-I, **III**). However, MANF positive cells were found within the Langerhans islet and pancreatic blood vessels of P1 *Pdx-1Cre*$^{+/-}$::*Manf*$^{fl/fl}$ due to incomplete recombination (Supplementary Figure 2.3K-O, **III**). Furthermore, mosaic immunoreactivity of MANF was observed in pancreases of *Pdx-1Cre*$^{+/-}$::*Manf*$^{fl/fl}$ at P14 with

the degree of variation, and even wider MANF expression was observed in adult conditional exocrine acinar and endocrine beta-cells (Figure 2F, Supplementary Figure 2.3A-I, **III**). Hence, diabetes in *Pdx-1Cre*$^{+/-}$::*Manf*$^{fl/fl}$ mice occur later than in *Manf*$^{-/-}$ mice possibly due to non-recombined islet cells expressing MANF.

Remarkably, certain mice did not show any MANF expression even in the adult age (Figure 2C, **III**). Accordingly, we observed a significant reduction of *Manf* mRNA levels in the islets derived from *Pdx-1Cre*$^{+/-}$::*Manf*$^{fl/fl}$ mice compared to *Manf*$^{fl/fl}$ at P1, P14 and P56 (Supplementary Figure 2.3J, **III**). Importantly, we detected an altered structure of endocrine islets and decreased expression of insulin-positive beta-cells in the pancreases of conditional mice (Figure 2A, 2J-O, **III**). When MANF expression was present in the beta-cells of the Langerhans islets, the morphology of the islet remained intact with the peripheral distribution of alpha-cells (Figure 2D, 2E **III**). We also noticed a loss of membrane expression of the glucose transporter 2 (GLUT2) in the beta-cells lacking MANF (Figure 3H-N, **III**).

Our results demonstrated that the number of MANF-positive beta-cells correlated with the beta-cell mass (Figure 2P, **III**), and consequently with blood glucose levels (Figure 2Q, **III**) in the *Pdx-1Cre*$^{+/-}$::*Manf*$^{fl/fl}$ mice. Also, a significant negative correlation was observed with the level of beta-cell apoptosis in individual mice (Figure 2R, **III**), further revealing that MANF is essential for the recovery of the beta-cell mass and maintaining the beta-cell phenotype.

5.3.2 Deletion of MANF specifically from beta-cells of adult mice

(Original publication **III**)

The analysis of *MIP1-CreERT*::*Manf*$^{fl/fl}$ mice injected with Tmx revealed no changes in the weight of these mice (Figure 5A, **III**). However, the blood glucose levels of the random fed *ad-libitum* mice were increased (Figure 5B, **III**), and insulin levels were significantly reduced (Figure 5C, Supplementary Figure 5.1A, **III**), as compared to oil injected mice. Physiologically, lack of MANF in adult beta-cells of Tmx injected *MIP1-CreERT*::*Manf*$^{fl/fl}$ resulted in impaired glucose tolerance *in vivo* shown by GTT (Figure 5D, Supplementary Figure 5.1B, **III**). ITT results indicated that insulin sensitivity was not abolished in Tmx injected *MIP1-CreERT*::*Manf*$^{fl/fl}$ animals (Supplementary Figure 5.1C, **III**), meaning that diabetic phenotype displays a deficiency of insulin production and/or secretion. Additionally, serum insulin levels after GTT were significantly downregulated in Tmx *MIP1-CreERT*::*Manf*$^{fl/fl}$ animals (Supplementary Figure 5.1D, **III**).

Moreover, deletion of MANF in the adult beta-cells led to reduced amounts of islets insulin content and as a result – decreased levels of glucose-stimulated insulin *in vitro* (Figure 5E-F, **III**), suggesting that the secretion pathway is functionally intact. However, in response to high glucose together with the 3-isobutyl-1-methylxanthine (IBMX), which is known to raise islet cAMP levels, the MANF-deficient islets secreted more insulin compared to islets from oil-injected mice. cAMP-stimulated insulin release is controlled by intracellular calcium (Siegel et al., 1980). As ER stress is known to release calcium from ER stores and increase cytosolic calcium, the higher insulin secretion from *MIP1-CreERT*::*Manf*$^{fl/fl}$ islets

might be due to ER stress leading to higher cytosolic Ca^{2+} which might potentiate the insulin secretion from the islets treated with IBMX.

Even though secreted insulin levels from islets of *MIP1-CreERT*::*Manf*$^{fl/fl}$ Tmx mice were reduced, the ability to secrete insulin in relation to total islet insulin content remained the same under glucose stimulation except for the condition with IBMX (Figure 5G, **III**).

Histological analysis of pancreases showed clusters of MANF-expressing cells (about 15% of the beta-cells) after the Tmx injection in *MIP1-CreERT*::*Manf*$^{fl/fl}$ mice (Figure 5H-M, **III**), which could be due to incomplete recombination and expansion of MANF-expressing beta-cells At the same time, we observed a nearly 50% reduction in *Manf* mRNA expression in the islets of oil injected *MIP1-CreERT*::*Manf*$^{fl/fl}$ mice, compared to *Manf*$^{fl/fl}$ islets , which could be due to leakiness of the system resulting in Cre-translocation into the nucleus even in the absence of Tmx. In accordance with reduced MANF IHC signal in the islets of *MIP1-CreERT*::*Manf*$^{fl/fl}$ mice, *Manf* mRNA levels were significantly reduced in isolated islets from Tmx injected *MIP1-CreERT*::*Manf*$^{fl/fl}$ mice (Supplementary Figure 5.1E, **III**).

Importantly, analysis of pancreatic tissue showed that the deficiency of MANF in the beta-cells of *MIP1-CreERT*::*Manf*$^{fl/fl}$ mice injected with Tmx caused beta-cell loss and decreased insulin expression (Figure 5O, **III**), a significant decrease of the beta-cell mass (Figure 5R, Supplementary Figure 5.1X, **III**), and a minor non-significant changes in the alpha-cell mass (Supplementary Figure 5.1Z, **III**) compared to oil injected control group. Additionally, we detected a significant reduction in beta-cell proliferation (Figure 5S, **III**) and a significant increase in apoptosis (Figure 5T, **III**) of beta-cells in Tmx *MIP1-CreERT*::*Manf*$^{fl/fl}$ mice. Moreover, we detected the reduced expression of PDX1 and GLUT2 in the adult beta-cells lacking MANF.

Thus, removal of MANF from the beta-cells postnatally led to the development of diabetes, as a result of reduced beta-cell proliferation and enhanced beta-cell apoptosis. This observation strongly suggests that MANF expression is needed for the beta-cell maintenance in adult mice.

5.3.3 Ablation of MANF in CNS specific mice does not cause a diabetic phenotype

(Original publication **II**, unpublished results)

The Nestin-Cre mouse model is commonly used to conditionally ablate or express particular genes in the neuronal cells of the CNS and PNS (Tronche et al., 1999). Although nestin is a marker for neuronal progenitor cells, its expression has been documented in several other tissues outside the CNS and PNS, including multipotent stem cells of the pancreatic primordium and kidney cells (Delacour et al., 2004; Dubois et al., 2006; Kedees et al., 2007). However, Cre-mediated recombination was observed in pancreatic exocrine cell lineage and not in the islet endocrine progenitor cells studied by cell lineage analysis (Delacour et al., 2004).

Recent study revealed that the level of MANF expression in the mouse hypothalamus is regulating food intake and thus insulin sensitivity (Yang et al., 2017) (chapter 1.4.4). However, the analysis of the random fed ad-libitum 1.25-1.5-year-old *NestinCre*$^{+/-}$::*Manf*$^{fl/fl}$

mice revealed no signs of diabetes as their blood glucose and serum insulin levels were not changed compared to control *Manf*$^{fl/fl}$ mice (Supplementary Figure 1J, 1K, **II**). Analysis of MANF expression in the pancreases of *NestinCre*$^{+/-}$::*Manf*$^{fl/fl}$ revealed patchy mosaic expression in exocrine tissue, but normal distribution in the islets of Langerhans (Figure 6). Consequently, histological analysis of the *NestinCre*$^{+/-}$::*Manf*$^{fl/fl}$ pancreases indicated intact islet structure with the peripheral distribution of alpha-cells (Figure 7).

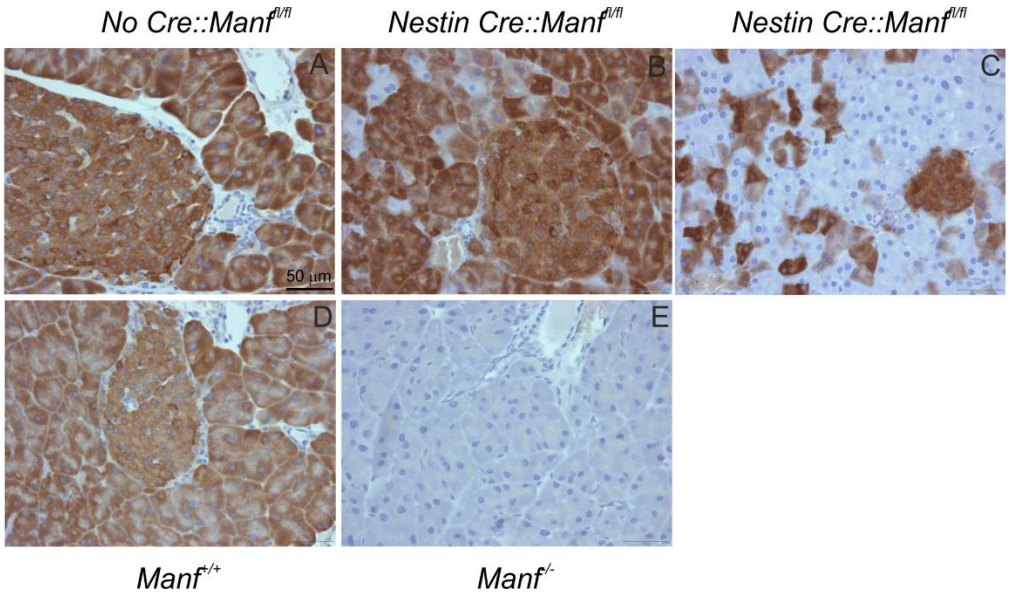

Figure 6. MANF expression is not affected in the islets of *NestinCre*$^{+/-}$::*Manf*$^{fl/fl}$ mice **(B,C)** compared to the *Manf*$^{fl/fl}$ **(A)**, *Manf*$^{+/+}$ **(D), and** *Manf*$^{-/-}$ **(E)** mice. Scale bar, 50 µm.

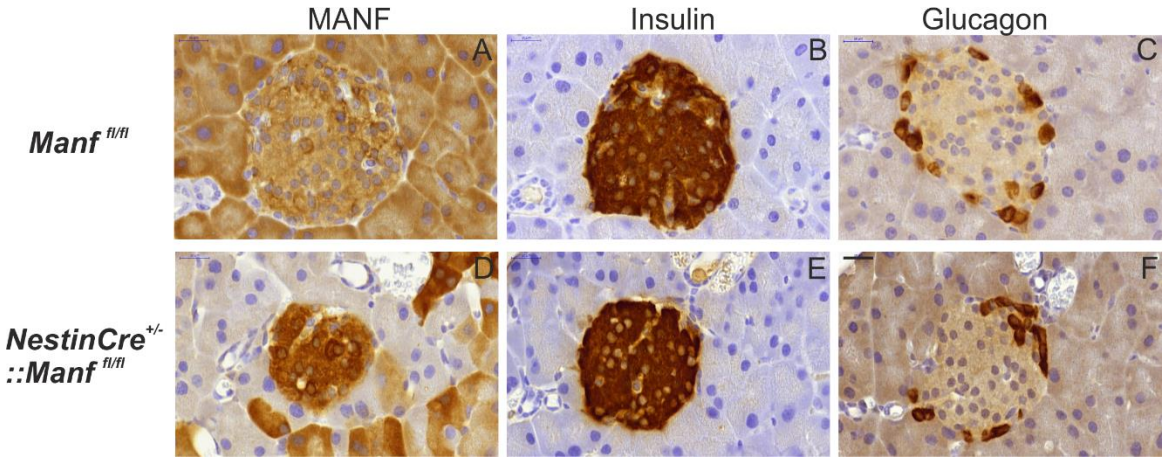

Figure 7. Structure of islets in *NestinCre*$^{+/-}$::*Manf*$^{fl/fl}$ mice is not affected. **(A-F)** MANF **(A, D),** insulin **(B, E)** and glucagon **(C, F)** immunohistochemistry on pancreas sections from *NestinCre*$^{+/-}$::*Manf*$^{fl/fl}$ and *Manf*$^{fl/fl}$ mice. Scale bar, 20 µm.

5.4 Activation of ER stress and UPR in the MANF deficient islets and pituitary glands

(Original publication **II**, **III**)

The mechanisms behind the reduced beta-cell mass and reduced number of acidophilic cells in the anterior pituitaries in MANF-deficient mice remained to be studied. As MANF has

been implicated in the regulation of the UPR and UPR is known to affect cell proliferation and lead, if chronic, to cell death, we set out to test level of UPR activation in the beta-cells and pituitaries of MANF-deficient mice. Pancreatic beta-cells are especially sensitive to ER stress due to their substantial need for insulin synthesis and secretion in order to regulate glucose homeostasis (Eizirik and Cnop, 2010). We observed chronic activation of all UPR branches and unresolved ER stress in the islets lacking MANF. First, we identified the upregulation of *Grp78* and *spXbp1* mRNA in the IRE1α pathway in the E18.5 pancreases from *Manf* $^{-/-}$ mice. In isolated pancreatic islets of *Manf* $^{-/-}$ mice at P1 and P14 (Figure 4A-C, **II**) we found increased activation of genes in the PERK and ATF6 pathway. Moreover, we observed increased levels of phospho-eIF2α in islets derived from MANF knockout mice at P14, P28, and P56 (Figures 4D, 4E, **II**), indicating constitutive activation of PERK pathway in the *Manf*$^{-/-}$ islets postnatally. The absence of MANF in the pancreas of *Pdx-1Cre*$^{+/-}$::*Manf*$^{fl/fl}$ mice leds to upregulation of UPR markers and chronic ER stress in pancreatic islets from P1 and onwards (Figure 3D, 3E, **III**). Importantly, the ablation of MANF in the adult beta-cells also resulted in the initiation of ER stress by a significant increase of *Grp78*, *Chop*, *Atf4*, *Atf6α*, and *Atf6β* mRNAs (Figure 6B, **III**). However, mRNA levels of *spXbp1* and *totalXbp1* were unchanged (Figure 6B, **III**), at the time of analysis 4 weeks after Tmx injections. Thus increased expression of *spXbp1* could have already preceded the upregulation of PERK and ATF6 pathways as in global *Manf*$^{-/-}$ and *Pdx-1Cre*$^{+/-}$::*Manf*$^{fl/fl}$ mice.

However, beta-cell mass, number of proliferating beta-cells and mRNA of different beta-cell markers were not yet reduced neither at E18.5 pancreases of *Manf* $^{-/-}$ mice nor in P1 islets of *Pdx-1Cre*$^{+/-}$::*Manf*$^{fl/fl}$ mice (Figure 3A, **II**, Figure 3A, **III**). The downregulation of the beta-cell mass and consequent decrease of *Glut2*, *Ins1/2*, *Pdx1* and *MafA* beta-cell markers were observed in the islets of *Manf* $^{-/-}$ mice starting from P1 and *Pdx-1Cre*$^{+/-}$::*Manf*$^{fl/fl}$ mice starting from P14 (Figure 3B, 3C, 3D, **II**, Figure 3B, 3C, **III**). Therefore, ER stress caused by MANF-deficiency seemed to precede the impaired beta-cell function.

Since the phenotype of *Manf*$^{-/-}$ mice is associated with the activation of ER stress and chronic UPR activation in pancreatic beta-cells, we investigated whether ablation of MANF from the pituitary gland leads to ER stress and activation of the UPR. Indeed, *Grp78*, *Chop*, *spXbp1, Atf4, Atf6α, Atf6β mRNAs* were significantly upregulated (Figure 6W, **I**), indicating severe chronic activation of all three central UPR pathways PERK, IRE1α and ATF6 in the pituitary glands of 6 weeks old *Manf*$^{-/-}$ mice lacking MANF. Consequently, as with the beta-cells, activation of those pathways seem to lead to reduced cell proliferation in the anterior pituitary. No increased cell death was observed in the anterior pituitaries of *Manf*$^{-/-}$ mice at 6 weeks, although apoptotic *Chop* mRNA levels were increased.

ER stress and activation of the UPR is associated with the pathogenesis of T1D and T2D (chapter 3.5.1 and 3.5.2) as well as diabetic phenotypes occur due to genetic alteration in several factors essential for the ER homeostasis (chapter 3.5.3). Similarly to MANF knockout mice, inactivation of several UPR genes in mice lead to the development of diabetes due to the loss of the beta-cells (chapter 3.5.3). Interestingly, similarly to *Manf*$^{-/-}$ mice *Perk* mutation in human and mice leads to skeletal dysplasia, growth retardation and diabetes (Zhang et al.,

2002). ATF4 is also involved in normal osteoblast differentiation and growth (Yang et al., 2004).

Taken together, MANF is an essential factor that regulates cell homeostasis and survival similarly to other UPR proteins.

5.5 Activation of signaling pathways in the islets of *Manf*$^{-/-}$ mice

(Original publication **III**)

MANF remains an orphan ligand, as its signal transduction receptor has remained elusive. So far, studies showed that MANF interacts with GRP78 and consequently regulates protein folding in the ER (Glembotski et al., 2012; Yan et al., 2019). The studies by Henderson et al. showed that MANF weakly binds to KDEL receptors (Henderson et al., 2013), although the direct binding was not confirmed. Recent studies identified that MANF binds to sulfatides at cell membranes of *C. elegans* and mammalian cells and this interaction enhances subsequent cellular uptake of MANF resulting in cytoprotection from hypoxia-induced cell death (Bai et al., 2018).

The exact signaling pathways activated by MANF remain undefinable. It was demonstrated that the PKC signaling cascade is activated after stimulation with 1.5 mg/ml of MANF in PC12 cells *in vitro* (Yang et al., 2014a). It was shown that MANF regulates NF-κB and p38 (MAPKs) pathways (Chen et al., 2015b; Zhu et al., 2016). Specific DmMANF knockdown in glia cells of *Drosophila* resulted in the appearance of a microglia-like cell type, which is characterized by expression of the NF-kB factor Relish (Stratoulias and Heino, 2015). Moreover, MANF was shown to protect neurons via the induction the PI3K/AKT signaling cascade (Zhang et al., 2017b; Gao et al., 2018; Xu et al., 2018). In contrast, reduced AKT phosphorylation was observed after increased expression of MANF in the hypothalamus of MANF-transgenic mice, leading to impaired insulin signaling, insulin resistance and hyperphagia (Yang et al., 2017).

We are the first to show that exogenous MANF can induce beta-cell proliferation and protect against ER stress-induced mouse and human beta cell death *in vitro* (chapter 6.1). However, the mechanisms involved in these processes are still unclear. In order to study the effects of exogenous MANF in beta-cells *in vitro*, we isolated islets derived from *Manf*$^{+/+}$ and *Manf*$^{-/-}$ mice and cultured the beta-cells in the presence or absence of MANF. Islet lysates were subjected to Western blotting using different sets of cell signaling antibodies or mRNA from islets was subjected to RT-qPCR. Upon addition of MANF to WT and KO islets, we observed an increase in AKT (Ser473) phosphorylation, suggesting that MANF exerts its proliferative effects on the beta-cells via PI3K/AKT cascade (Figure 7A, 7B, **III**), which is one of the important pathway involved in mitogenic responses in rodent and human beta-cells and can be initiated by many growth factors like insulin, IGF-1, HGF, GLP-1 (Elghazi and Bernal-Mizrachi, 2009; Szabat et al., 2016). ERK/MAPK signaling pathway is also known to promote beta-cell replication and can be initiated by PDGF, EGF receptor and insulin receptor (Cras-Meneur et al., 2001; Chen et al., 2011; Siddle, 2011). However, we did not observe activation of the ERK pathway in the mouse beta-cells upon addition of MANF (Figure 7E, 7F, **III**).

Recent study indicated that PDGF-mediated MANF expression stimulated anti-inflammatory effects in the injured retina, thus leading to retinal tissue repair in mice and fruit flies (Neves et al., 2016). The analysis of the signaling cascades in MANF deficient islets revealed activation of pro-inflammatory pathways including p38MAPK, NF-κB and JNK (Figure 7G, 7H, 7K, 7L, 7M, 7N, **III**), initiation of which results in deleterious effects of the beta-cells (chapter 3.5.1). Studies with human beta-cells revealed that MANF protective properties are associated with the repression of the NF-κB signaling pathway (Hakonen et al., 2018). Moreover, treatment of primary human islets with cytokines and MANF led to downregulation of apoptotic BCL10 (Hakonen et al., 2018), which binds to TRAF2 thereby leading to apoptosis and activation of NF-κB (Yoneda et al., 2000). Consequently, we detected that *Bcl10*mRNA levels were significantly enhanced in *Pdx-1Cre*$^{+/-}$*::Manf*$^{fl/fl}$ islets at P1 (Figure 7I,7J, Supplementary Figure 6B, **III**). Moreover, we detected enhanced expression of iNOS in the *Manf*$^{-/-}$ islets (Figure 7O, 7P, **III**), thus possibly leading to NO production. Additionally, upregulation of c-Jun phosphorylation in KO islets was detected (Figure 7K, 7L, **III**). Hence, the protective effects of MANF can be mediated by immune modulation.

Furthermore, increased levels of *Trib3* mRNA was detected (Figure 7C, 7D, **III**), which is a negative regulator of AKT phosphorylation (Du et al., 2003). It is known that ATF4 in the PERK-pEIF2α pathway regulates the expression of Trib3 (Cunard, 2013), thus the reduced phospho-AKT in MANF-deficient islets could be caused by increased ATF4 expression that upregulates the expression of Trib3 leading to increased inhibition of AKT phosphorylation (Figure 7A, 7B, **III**). Hence, we could speculate that the reduced AKT-phosphorylation in *Manf*$^{-/-}$ beta-cells leads to decreased beta-cell proliferation due to chronic activation of the UPR and specifically the PERK pathway. In addition, increased inflammatory signaling leading to beta-cell death of *Manf*$^{-/-}$ islets might be caused by increased IRE1α and PERK activation. Consequently, MANF might dampen the ER stress in beta-cells thus favour beta-cell proliferation and protection.

The summary of the signaling pathways activated in response to MANF deficiency in the beta-cells provided in the Figure 8.

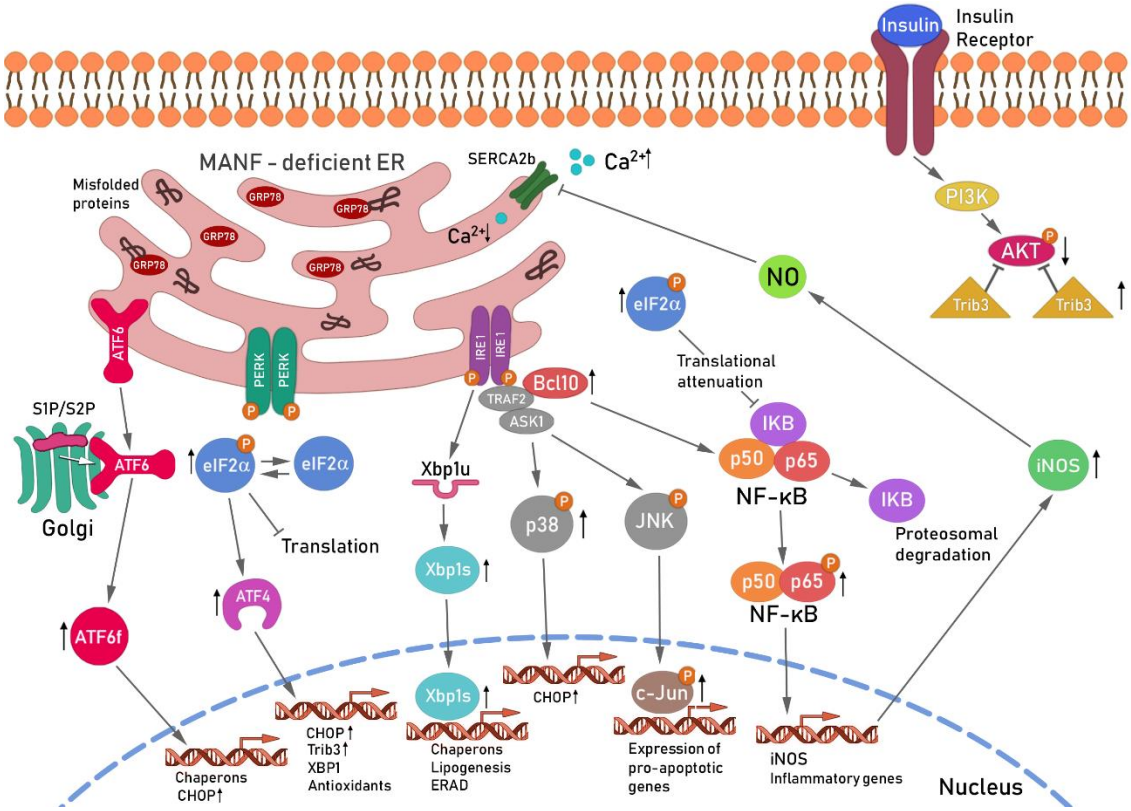

Figure 8. Illustration of signaling pathways activated in response to MANF deletion from mouse pancreatic islets based on results provided in chapters 5.4 and 5.5. Chronic activation of all three central UPR pathways PERK, IRE1α and ATF6 were observed in pancreatic islets deficient to MANF that was accompanied by the activation of pro-inflammatory signaling cascades including p38MAPK, NF-κB and JNK. Levels of pro-apoptotic *Bcl10* were significantly enhanced as well as iNOS expression. Increased expression of *Trib3* resulted in inhibition of AKT phosphorylation in MANF-deficient islets. ATF6, activating transcription factor 6; PERK, protein kinase R-like endoplasmic reticulum kinase; IRE1, inositol-requiring enzyme 1; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; JNK, c-Jun N-terminal kinases, iNOS, inducible nitric oxide synthase: NO, nitric oxide; BCL10, B-cell lymphoma/leukemia 10; TRIB3, tribbles homolog 3. Modified from (Danilova and Lindahl, 2018).

6. Therapeutic effects of MANF

As therapeutic strategies for the development of new molecular regulators of endogenous beta-cell regeneration is under intensive investigation for the treatment of diabetes (Vasavada et al., 2006; Tarabra et al., 2012), we set out to study the regenerative effect of MANF *in vitro* on primary mouse beta-cells as well as *in vivo* in a mouse model of T1D.

6.1 Exogenous effect of MANF on primary mouse beta cells

(Original publication II, III and (Cunha et al., 2017))

Based on the result that *Manf*$^{-/-}$ mice have reduced beta-cell proliferation compared to WT mice, we speculated if MANF protein could affect the replication of the mouse beta-cell in culture. Our results demonstrated that MANF can stimulate beta-cell proliferation in isolated islets *in vitro* prepared from young adult (12 weeks of age) (Figure 5A, **II**). Notably, MANF

in combination with PL showed an additive effect in the proliferation efficiency on young adult mouse beta-cells implying different mitogenic intracellular signaling cascades for MANF from PL, which is known to stimulate beta-cell replication trough Janus kinase-signal transducer JAK2/STAT5 pathway (Amaral et al., 2003).

Expansion of the beta-cell mass occurs under the process of beta-cell proliferation postnatally (Dor et al., 2004). However, replication of the beta-cells deteriorates in rodents and humans with advanced age as well as the ability of aged beta-cells to respond to the mitogenic factors (Teta et al., 2005; Kushner, 2013). Importantly, we showed that MANF enhances the proliferation of the beta-cells of even aged mice (15-month-old mice) (Figure 4A, **III**). With collaborative efforts, we demonstrated that MANF in combination with TGFβ promoted enhanced primary human beta-cell proliferation (Hakonen et al., 2018), which rarely replicate. Taken together, the MANF is capable to stimulate replication of the beta-cells from even aged mice and humans imply that MANF is a mitogenic factor for pancreatic beta-cells.

Furthermore, our studies evaluated the ability of MANF to inhibit beta-cell apoptosis under several ER stress conditions. We showed that thapsigargin upregulated *Manf* mRNA expression in mouse beta-cells in vitro and that MANF was able to decrease thapsigargin-induced apoptosis (Cunha et al., 2017) (Figure 9). In addition, treatment of mouse beta-cells in media supplemented with high glucose significantly upregulated *Manf* mRNA *in vitro* (Figure 4K, **III**) (Figure 9), suggesting that hyperglycemia in mice and human leads to upregulation of MANF expression in the beta-cells *in vivo*. MANF able to decrease hyperglycemia-induced ER stress in mouse beta-cells *in vitro* (Figure 4L-N, **III**).

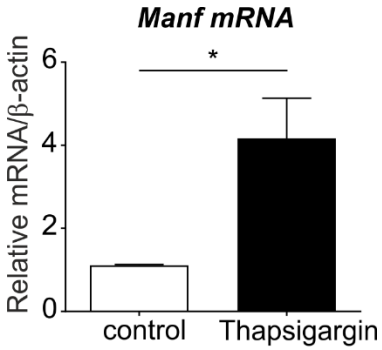

Figure 9. Quantitative RT-PCR analysis of *Manf* gene expression in primary mouse pancreatic islets treated with 1 μM thapsigargin for 6 hours in RPMI medium supplemented with 0.5% BSA. *n* = 2 wells per group.

Finally, we showed that the addition of MANF to the MANF-deficient islets in the culture medium led to increased beta-cell proliferation (Supplementary Figure 4A, **III**) and reduced levels of ER stress markers compared to untreated islets (Supplementary Figure 4B, 4C, **III**).

Our collaborative studies demonstrated that MANF is secreted from in human beta-cells upon stimulation with pro-inflammatory cytokines *in vitro* (Hakonen et al., 2018). Importantly, MANF partially protected both mouse and human beta-cells from cytokine and ER stress-induced cell death *in vitro* (Cunha et al., 2017; Hakonen et al., 2018). Pathogenesis

of T1D is associated with the production of pro-inflammatory cytokines that trigger activation of ER stress and UPR in the beta-cells (chapter 3.1 and 3.5.1), therefore MANF is especially essential factor T1D therapy.

To conclude, our results show that MANF is an essential factor with mitogenic and defensive properties for both mouse and human beta-cells. Hence, the effects of MANF *in vitro* make it a promising target for the treatment of diabetes.

6.2 Adeno-associated virus (AAV) vector-mediated overexpression of MANF in the pancreases by intraductal delivery partially protects beta-cells in STZ mouse model

(Original publication **II**)

To study the therapeutic potential of MANF on mouse beta-cells *in vivo*, we proceeded with intraductal delivery of adeno-associated virus vector serotype 6 (AAV6) -MANF and AAV6-RFP as a control into the pancreases of mice, followed after 3 weeks by multiple low-dose streptozotocin (MLDS) injections in order to induce diabetes in mice (Figure 5B, **II**). STZ is a toxic glucose analog that selectively enters rodent beta-cells via GLUT2 and subsequently kills beta-cells (Schnedl et al., 1994). MLDS is generally used in experiments attempting to reproduce mild insulin-dependent diabetes mellitus in rodents. For accurate study design, we considered performing STZ injections in 3 weeks after the intraductal delivery of AAV6-MANF and AAV6-RFP in order to let MANF and RFP overexpress (Figure 5B, **II**).

Mice were monitored for blood glucose levels and sacrificed 3 weeks after the first STZ injection when control mice were highly hyperglycemic. The blood glucose and serum insulin levels were similar between the control and treatment groups indicating that MANF did not have significant therapeutic effect in this experiment (Supplementary Figure 5M, 5N, **II**). Histological analysis of the pancreases of these mice revealed that only about 4% of beta-cells and exocrine tissue were transduced with the virus, indicating a partial delivery and over-expression of MANF and RFP (Supplementary Figure 5A-K, **II**), which could explain the negative therapeutic effect. Nevertheless, we observed that insulin-positive islets were significantly more abundant and larger in the AAV6-MANF-STZ-treated group compared to the AAV6-RFP-STZ-treated mice (Figure 5C, Supplementary Figure 5D, 5H, 5L, **II**). Importantly, overexpression of MANF in the pancreas seemed to increase beta-cell proliferation but not exocrine acinar cell proliferation measured by Ki67 IHC and protect from beta-cell death assessed by TUNEL in our MLDS mouse model of diabetes (Figure 5D, 5F, **II**).

Hence, overexpression of MANF in beta-cell demonstrated regenerative and protective effects on beta-cells in MLDS mouse model of T1D. Taking into account the limitations of this study such as low-transduction efficiency of virus-delivered MANF, more research is required to address the therapeutic potential of MANF in diabetes. We have thus produced beta-cell-specific MANF-overexpressing transgenic mice which will be used to further validate the role of MANF-overexpression in mouse diabetes models.

7. Morphological analysis of pancreatic tissues with deep convolutional neural network

(Manuscript **IV**)

Deep convolutional neural network (dCNN) based algorithms are a very effective method in the objects and patterns recognition, which is successfully employed in medical and biological research. dCNN is applicable to use for cell areas quantifications, as well as, individual cells with the advantages in reduced human errors, decreased variability, and faster analysis, which all increase the analysis capacity. We implemented the analysis of the mouse whole pancreatic sections immunolabeled with either insulin or glucagon antibody to count beta- and alpha-cell areas following by quantification of the beta-, alpha- and islet-cell mass. Importantly, by using dCNN we obtained islet-cell areas results from the slides stained with either insulin or glucagon antibodies. Next, we compared the results of dCNN method to our previously published results using pancreatic sections from diabetic conventional and conditional MANF-deficient mice and their controls. Our data demonstrated that dCNN for the morphological analysis of the mouse pancreatic tissue is a highly accurate method. We obtained a significant correlation between the results of the traditional method and the dCNN algorithms. The new approach will noticeably increase the analysis performance and generate high-quality data from pancreatic sections examination (in average 1 minute 4 seconds for the analysis of 1 pancreatic section, which is about 6x times faster than the analysis done by manual methods).

SUMMARY AND CONCLUSIONS

The main aim of this thesis was to investigate the biological functions of MANF *in vivo*. The following findings were presented in this thesis:

I. Extensive expression analysis of MANF protein and mRNA in different mouse tissues revealed that MANF is highly expressed in the mouse tissues with metabolic function, especially in glandular cells with the high secretory function.

II. We showed that the global lack of MANF leads to insulin-dependent diabetes and growth retardation in mice. The diabetic phenotype of *Manf*$^{-/-}$ mice was caused by progressive postnatal loss of beta-cell mass due to the postnatal decrease in the beta-cell proliferation and increased apoptosis. The growth defect in MANF-deficient mice could partly be caused by the reduced size of the anterior pituitary gland and decreased number of growth hormone producing cells.

III. Embryonic ablation of MANF specifically from the pancreas in *Pdx-1Cre*$^{+/-}$::*Manf*$^{fl/fl}$ conditional knockout mice resulted in the loss of beta-cells and diabetes similarly to *Manf*$^{-/-}$. However, incomplete recombination and mosaic MANF expression in postnatal pancreases of *Pdx-1Cre*$^{+/-}$::*Manf*$^{fl/fl}$ mice revealed a significant correlation between the number of MANF-expressing beta-cells and beta-cell mass and consequently with blood glucose levels in individual mice demonstrating the significance of MANF expression for maintaining the beta-cell mass.

IV. Excision of MANF from beta-cells of adult mice by using tamoxifen-inducible *MIP1-CreERT::Manf*$^{fl/fl}$ mice led to the development of diabetes associated with decreased beta-cell mass caused by increased beta-cell apoptosis, possible de-differentiation of the beta-cell and reduced beta-cell proliferation. Thus, MANF expression in beta-cells is required for the maintenance and survival of adult beta-cells in mice.

V. MANF-deficiency in pancreatic beta-cells and pituitary glands leads to ER stress and chronic UPR activation *in vivo*.

VI. We identified that PI3K/Akt pathway is implicated in the MANF signaling. Our experiments show that MANF protein mitigates ER stress in *Manf*$^{-/-}$ islets in culture. Moreover, MANF-deficiency in cultured islets leads to the activation of pro-inflammatory NF-κB, p38, JNK signaling cascades. These results might indicate that MANF could rescue beta-cells from apoptosis by relieving ER stress thus decreasing inflammatory signaling.

VII. Recombinant MANF protein increased beta-cell proliferation *in vitro* in islets isolated from young and aged mice. Moreover, MANF protected mouse beta-cells from ER stress-induced beta-cell death triggered by thapsigargin and hyperglycemia-induced ER stress *in vitro*. Thus, MANF showed mitogenic and protective effects for mouse beta-cells.

VIII. We also described a new fast and fully automated graphical software that assesses the areas of islets, beta-cells, alpha-cells and exocrine tissues of the pancreas based on the dCNN technique.

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